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Physiological Study on the Adaptability of Rice Plants to the Water Logged Soil Condition

Part 1. On the flooding induced chlorosis of rice seedlings*

Sei-ichi TAKAGI

(Received on January 25, 1960)

I. Introduction

Rice plants generally grow better in water logged soil than in upland soil. Such a character of theirs is not only a matter of biological interest to clarify, but also one of the fundamental problems in the cultural technique. This is the reason why not a few studies have long been made from various angles, that is, from their respiration, mineral nutrition, water relation, etc. Even at present, however, the results of these studies still remain at a stage where they are only isolated pieces of knowledge as to the physiology and morphology of rice plants.

First of all, a general survey must be given to the character of water logged soil conditions, under which rice plants grow, in order to systematize these pieces of knowledge, and then the following recognition might be admitted in this respect. That is to say, the first to be mentioned as a difference between the water logged soil and upland soil condition is "Water logging", in other words, a difference in the water environment. The second is that as an inevitable result, the airface is displaced, and that the supply of the oxygen for the plant root and soil microbe is excessively held in check. Furthermore, as a result of this phenomenon, is caused a change in the chemical condition in the soil. This is the third character.

Consequently, the better growth of rice plants under water logged soil condition can be attributed to their adaptation to the associated condition of the 3 characters. From this fact it can be expected that there may be a close relation among the adaptation mechanisms of the same plants to each of the characters. Next, the author's opinion on the studies that have ever been made on the subject concerned will be given, based on the above standpoint.

Onodera[1] Ueda[2] Kishida[3] and many others reported on the effect of soil moisture content on the physiology and morphology of rice, and Yu. S. V. *et al.*[4], has recently examined the physiological difference in drought resistance between lowland and upland rice. According to these results, it is almost certain that the meaning which water logging bears in the paddy fields is found mainly in an advantage of supplying moisture.

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These studies, however, were all made, with natural soil as their media, and therefore some effects of other factors can not be neglected in these cases, that is, there still is left a doubt that water logging might contribute rather to supplying nutrients than moisture itself. For example, Kobayashi[5] reported on the increase in the yield and better growth of upland rice by controlling the nitrification of soil. And Lin[6] and Ponnamperuma[7] insisted that the advantage of water logging comes chiefly from the increase in supplying iron ; in their conclusions, however, the effect of moisture condition was completely neglected. Judging from the above mentioned problems, it is necessary, after all, to re-examine under the condition, with each of these two factors controlled independently, the problem as to whether flooding in soil contributes chiefly to supplying moisture or nutrients.

Next, the characters of rice plants must be mentioned from another point of view. They respond to the complicated change of the chemical condition in water logged soil. The prominent phenomena among them are probably their requirement of much iron,[6),10),23–25] their preference for ammonium to nitrate as their nitrogen source,[11–13] and their strong root's power oxidizing reducing substances.[14]

It is especially noteworthy that in most of the studies of the kind, experiments were made under the water culture condition. Viewed from the water environment, however, the water culture condition is nothing but a kind of water logging condition. Consequently, it can be pointed out that not a study has ever been made on the above characters discerned under such water environment as the water culture condition, in order to clarify what change will take place in those characters under the water environment in upland soil.

It has lately been suggested that the upland nursery seedlings require nutrients different from those which are required by lowland nursery seedlings. That is to say, Yamada and Ōta[15–17] proved that the former contain a large quantity of starch in spite of the high nitrogen content, and that the latter contain comparatively much manganese with the result that iron is prevented from being transported into leaf blades. Their report also says that such characters of the seedlings at their nursery stage can be discovered in their state of nutrient absorption after they are transplanted. Besides, Moriya and Satō[18] reported that the application of more phosphorus is needed for the suitable growth of upland nursery seedlings than for that of lowland ones.

Such a change in the nutrients requirement of rice plants are supposed to be caused by one or more of such factors as water, oxygen, and nutrients as was mentioned above. The analysis of this problem has not yet been made.

What was mentioned above convinces the author that it is quite necessary to determine physiological significance of each of the factors such as water, oxygen and nutrients.

Based on the above conclusion, the author tried a comparison of the growth responses revealed under the moisture conditions of upland and flooded, with synthetic soil as the medium in order to prevent the change in nutritional condition brought about by the change in soil moisture. And as a result, he found that rice plants are more susceptible of iron deficiency chlorosis, — that is, a distinct phenomenon to be called flooding induced chlorosis, under the condition of water logging than under the upland moisture condition. In the present paper, various factors relative to the emergence of such iron chlorosis were examined, and in addition some considerations were given to the meanings of the phenomenon from the view point of the adaptability of rice plants to submerged soil condition.

II. Methods

1. Preparation of medium

The synthetic soil used for the experiments was washed sand mixed with clay minerals. The sand was air-dried after colloidal fractions had been removed as far as possible by the repetation of washing river sand as it was stirred. The clay minerals were a mixture of bentonite and acid earth prepared so that they show the approximate values of pH 7. The quantity for a pot was 2.2 kg of the sand and 50 g of the clay minerals, white painted cans with a capacity of 2 liter being used as containers (pots).

Ammonium nitrate and potassium biphosphate were used for fertilizer, the quantity per pot being N : 280 mg and P : 155 mg. The pH of the synthetic soil was approximately 6.8, and sulfulic acid or calcium hydroxide was used for the control of pH. The base exchange capacity (N-ammonium acetate method) of washed sand and clay mixture being 3.4 and 56.7 me/100 g respectively, and their organic matter and total nitrogen only a trace.

2. Cultural method

The synthetic soil thus prepared was placed in the pots, and watered till it showed about 70 % of its full water holding capacity. After leaving them as they were for a few days, germinated seeds were sown, and moisture condition designed was given to the plants from the time when the first leaves finished development.

The control of the moisture content was carried out by measurement of the weight of the pots, that is, they were watered every day. The absolute amount of water under the full water holding capacity of the medium being 500 g per pot. As a general rule, the standard moisture content in the case of upland conditions was 70 % of the full water holding capacity, and that in the case of water logging 130 %. The water layer on the soil surface of the latter was 1–2 cm in thickness. The experiments were made by the triplicate method.

As the auhor reported in the previous paper,[19] the discharge of orgainc matter from roots increases even in such medium of synthetic soil, when the growth of rice reaches a certain stage, and as a result reduction takes place in the medium. To avoid such an effect, the plants were harvested before their fifth leaves completed development.

3. Harvesting and determination of chlorophyll content

In harvesting the sand were removed from the roots with running water, the top parts cut off the roots, dried at 80°C and then their air dry weight measured after they were left few days in the dark to make them attain equilibrium with the atmospheric humidity.

For the determination of the chlorophyll content of blades, extraction was carried out for 48 hours in a dark place at 20°C. with 10 ml of 80% acetone added per 100 mg of the dry material, the solution shaken occasionally. Next, the extract was diluted by adding 10 ml of 80 % acetone per 1 ml, and the relative values of chlorophyll content was measured by reading the optical density (– Log T) on the conditions of the wave length 663 mμ and solution layer 10 mm.

III. Results

Experiments 1

In the preliminary experiment rice seedlings were grown with the above mentioned synthetic soil as a medium under the moisture conditions of upland and flooded. As a result, it was observed that they grew normally under the former condition, while chlorosis emerged under the latter. As the chlorosis seemed to be iron deficiency chlorosis from its symptom, the following experiment was tried for the purpose of ensuring this point and further of the examination of its cause.

The plan of each experiment will be mentioned next, the variety used being lowland rice. var. *Nōrin No. 16*, and in these treatments two comparable series of upland and water logging designed.

(A) On the effect of medium pH

The pH of the media were contrived to show the following 3 levels : 6.0 (got by adding 5 ml of 2-N sulfuric acid per pot), 6.8 (non-treatment) and 8.1 (got by adding 1.0 gram of calcium hydroxide per pot). The number of seeds sown was 18 per pot.

(B) Effect of chelated iron application

The application of chelated iron to the media and to the leaves was respectively carried out to the plants grown the same manner as in the treatment of pH 6.8 in (A).

(C) Effect of planting density

The number of the seeds was decreased to 3, the other treatments being the same as in the case of (A).

(D) Effect of phosphorus levels of medium

For this experiment was used the medium of pH 6.0, and the amount of phosphorus applications designed so as to show three levels, such as 0, 155 and 620 mg respectively. The present experiment was different in the growing conditions from the others, so that a quantitative comparison was difficult between the present result and each of the three experiments (A), (B) and (C).

(E) Experiment on barley

For the comparison with the results obtained from the rice, an experiment was made on barley (var. *Aizu No. 2*). Grains sown per pot was 8 in number.

They were cultured in a glass house. Rice seeds were sown on Sept. 25th 1958, and those of barley on Sept. 27th. On Oct. 2nd when they almost developed their first leaves, flooding was begun in the flooding series, and mositure continued to be kept as much as 70% of the full water holding capacity in the upland series. Most of the rice plants developed their 2nd leaves on Oct. 10th, when chlorosis could be obsesved on the 2nd leaves of those in the treatment plot of pH 8.1 belonging to the upland series, and of those in most treatments in the flooding series.

The degree of chlorosis in the latter series, however, was conspicuous in the treatments of pH 6.7 and 8.1, while it was slight in that of pH 6.0, and in the case of no phosphorus, such a phenomenon could not recognizable. So far as the barley is concerned, chlorosis could not be discovered in all the treatments, though they reached the leaf stage of about 2.2.

In the treatment whose pH was 6.8, the rice plants in the upland series reached the leaf stage of 2.9 on Oct. 18th, while those in the flooding series the stage of only 2.5, because the leaves were late in coming out owing to chlorosis. On the same day, the application of chelated iron was carried out to the media and to the upper most leaves in both the series.

For soil application were used EDTA-Fe and DTPA-Fe, both prepared so as to show pH 6.9 and 0.2 mol in concentration, 30 ml of which were respectively added per pot. And as to the method of application to the leaf surface, the EDTA-Fe solution, the same as that used for soil application was applied to the developed parts of the 3rd leaves, with a small quantity of steicker in it. EDTA-Na solution whose pH and concentration were the same, were used for the control.

About 3 or 5 days after the treatment the leaves applied with the EDTA-Fe showed distinct greening only on the parts applied with the solution.[20),21)] In the treatment of soil application of these two kinds of chelated iron, complete greening could be observed in the whole plants, and a rapid growth was re-

commenced. On the contrary, no effect was recognized on the leaves to which the EDTA-Na was applied.

Harvest was gathered on Oct. 27th of the rice grown in the 3 experiments, (A), (B) and (C) ; in the experiment (D) on 29th and in (E), experiment on barley, on 18th of the same month. In Table 1 (a), (b), and (c) are shown the air dry weight of tops and chlorophyll content of the leaf blades obtained from the harvest in each of the experiments (A), (B) and (C).

Table 1. Effect of medium pH, planting density and the application of chelated iron to the medium, on the chlorophyll content and dry matter production of lowland rice *Nōrin-16* ; especially on the difference in the effect between the upland and flooding moisture conditions of synthetic soil media.

Moisture condition in the medium	Number of plants per pot	Medium pH		Air dry weight of plant		Chlorophyll content of leaf blade		
		At the sowing	At the harvest	Top	Root	2nd leaf	3rd leaf	4th leaf
		(a)						
				(mg)	(mg)	(−LogT)*		
Upland	24	6.0	5.9	90.6	11.6	0.482	0.572	0.474
〃	〃	6.8	6.2	84.0	11.0	0.432	0.472	0.346
〃	〃	8.1	6.9	61.5	12.7	0.316	0.262	0.210
Flooding	24	6.0	6.0	70.6	8.7	0.304	0.288	0.312
〃	〃	6.8	6.5	34.3	6.3	0.086	0.074	0.096
〃	〃	8.1	7.4	35.8	6.5	0.132	0.080	0.062
		(b)						
Upland (Fe·EDTA application)	24	6.8	6.1	81.9	9.9	0.360	0.496	0.428
〃 (Fe·DTPA application)	〃	6.8	6.1	84.7	9.7	0.400	0.510	0.436
Flooding(Fe·EDTA application)	24	6.8	6.1	57.0	7.5	0.278	0.476	0.350
〃 (Fe·DTPA application)	〃	6.8	6.1	50.0	7.2	0.228	0.434	0.322
		(c)						
Upland	3	6.0	6.1	104.2	11.6	0.360	0.490	0.416
〃	〃	6.8	6.2	90.8	10.4	0.280	0.230	0.292
〃	〃	8.1	6.7	61.4	10.1	0.180	0.222	0.208
Flooding	3	6.0	6.1	85.0	11.4	0.246	0.270	0.332
〃	〃	6.8	6.7	37.5	5.3	0.110	0.088	0.126
〃	〃	8.1	7.7	34.4	4.7	0.080	0.074	0.020

* Optical dencity of acetone extract of leaf blade (at the 663 mμ).

In Table 1 (a), the chlorophyll content of the leaf blades decline in quantity in proportion to the increase in the medium pH, in the cases of both upland and flooding series. And when the pH in the media are equal in value, the content is excessively smaller in quantity in the flooding series than those in the upland series.

The air dry weight shows a tendency almost similar to that of the chlorophyll content. This probably suggests within the range of the present experiments that chlorophyll content plays a part of the major limiting factor to the dry matter production of rice.

The pH of the media at the time of harvest, in general, is higher in the treatments belonging to the flooding series than those in the upland ones showing

the comparable pH. It is, however, impossible that such a difference in the pH is the cause which brought about the chlorosis of the rice in water logging. The reason is that the difference in the final pH between both the series is not vast enough to be regarded as the cause of the difference in the quantity of chlorophyll content. In some other instances, moreover, some final pH values of the media in the flooding series were lower than in the upland ones (cf. table 4).

Then the effect of the soil application of chelated iron is considered in Table 1 (b). Chlorophyll content increases greatly in the flooding series, and so does the air dry weight in proportion to the increase in the former. In the case of the upland series, the former indicates a slight increase, while no effect is observed in the latter.

The above results demonstrate that the chlorosis accompanied with water logging is due to iron deficiency, and consequently that rice are more susceptible of iron deficiency under the flooding condition than under the upland moisture condition.

Next, let the author examine the difference in the chlorophyll content of the leaf blades between 24 and 3 seeds planted per pot, by comparing (a) with (c) of Table 1. Then it is certain that no clear difference can be found between those in the flooding series. This makes him suppose that the chlorosis accompanied with water logging is not caused by the change in the conditions of the media owing to the functioning of the plant root. In the upland series, on the contrary, chlorophyll content is clearly small in the case of 3 grains. This is thought to suggest that the effect of the root functioning upon the medium contributes to iron utilization under the condition of uplant moisture.

The result of the experiment (D) on the effect of phosphorus levels are shown in Table 2. In the flooding series, the chlorophyll content of leaf blades was proper and the plants also grew better in the case of 0, and 155 mg-P per pot. In the case of 620 mg-P, on the contrary, chlorosis emerged, and consequently dry matter production and leaf emergence velocity decreased. In the upland series, however, the plants presented phosphorus dificiency symptom, which

Table 2. Effect of moisture condition and phosphorus levels of the synthetic soil medium on the chlorophyll content and dry matter production of the lowland rice *Nōrin-16*.

Moisture condition in the medium	Phosphorus applied per pot	Medium-pH		Air dry weight of plant		Chlorophyll content		Leaf stage
		At the sowing	At the harvest	Top	Root	3rd leaf	4th leaf	
	mg			(mg)	(mg)	(−LogT)*	(−LogT)*	
Upland	0	6.0	6.4	69.0	11.9	0.562	0.506	3.7
〃	155	〃	6.0	84.2	10.5	0.624	0.542	4.1
〃	620	〃	6.3	80.8	9.2	0.546	0.472	4.1
Flooding	0	6.0	5.8	93.8	13.3	0.598	0.508	4.1
〃	155	〃	6.0	92.0	10.9	0.538	0.476	4.1
〃	620	〃	6.2	47.3	6.5	0.192	0.192	3.6

* Optical dencity of acetone extract of leaf blade (at the 663 mμ).

decreased their leaf emergence velocity in the case of 0 mg-P. In the case of 155 and 620 mg-P, on the contrary, they grew better. That is to say, there is a contradictory tendency in the emergence of iron and phosphorus deficiency with regard to rice between the upland and flooding series.

Table 3 shows the results of the experiment (E) on barley made for the comparison with those obtained from rice. So far as the present experiment is concerned, any symptoms of the chlorosis could not be recognized in the case of barley. And air dry weight was generally lower to a small degree in the flooding series than in the upland series. Further description will be given to the barley in Experiment 4.

Table 3. Effect of pH and moisture condition of the synthetic soil medium on the chlorophyll content and dry matter production of the barley *Aizu-2*. (8 plants per pot)

Moisture condition in the medium	Medium-pH		Air dry weight of plant		Chlorophyll content	
	At the sowing	At the harvest	Top	Root	2nd leaf	3rd leaf
			(mg)	(mg)	(−LogT)*	(−LogT)*
Upland	6.0	6.1	95.3	14.9	0.666	0.424
〃	6.8	6.6	92.7	13.6	0.574	0.441
〃	8.1	7.3	90.4	14.3	0.582	0.372
Flooding	6.0	6.3	77.3	13.5	0.584	0.420
〃	6.8	6.9	83.8	13.7	0.534	0.382
〃	8.1	7.5	81.7	13.8	0.596	0.376

* Optical dencity of acetone extract of leaf blade (at the 663 mμ)

Experiment 2.

In Experiment 1 (A), an experiment on the effect of medium pH, the observation of the emergence of chlorosis accompanied with water logging in a comparatively high pH range was aimed at, and consequently the optimum pH of the medium could not be obtained with regard to the chlorophyll content and the dry matter production of rice plant. In the present experiment the effect of pH within the comparatively low pH range were examined for the purpose of detecting the respective optimum pH under the water logging and upland conditions.

Lowland rice used was var, *Nōrin No. 16*, the method adopted being the same as that of Expt. 1 (A) except the following contrivances. That is, 5 levels of medium pH, 6.9, 6.4, 5.9, 4.9 and 4.5 were prepared. The plants were seeded on June 6th, 1959; flooding treatment was begun when their first leaves had been developed; and harvest reaped on July 1st at the approximate time of their 4.5 leaf stages.

Table 4 and Fig. 1 indicate the results obtained from the harvest. The chlorophyll content of leaf blades increases most abruptly in the range from pH 6.4 to 5.9 in the case of the flooding series, exhibiting a tendency to go on

Table 4. Effect of pH and moisture condition of the synthetic soil medium on the chlorophyll content and dry matter production of the lowland rice *Nōrin-16*. (16 plants per pot)

Moisture condition in the medium	Medium-pH		Air dry weight of plant		Chlorophyll content (4th leaf)
	At the sowing	At the harvest	Top	Root	
			(mg)	(mg)	(−LogT)*
Upland	6.9	6.9	126.6	14.6	0.502
〃	6.4	6.7	122.9	13.1	0.567
〃	5.9	6.2	119.8	12.3	0.540
〃	4.9	5.6	120.5	14.0	0.495
〃	4.5	4.7	106.5	11.9	0.481
Flooding	6.9	6.7	78.5	9.6	0.119
〃	6.4	6.4	101.5	11.9	0.182
〃	5.9	6.1	137.9	16.2	0.452
〃	4.9	4.9	143.4	16.4	0.519
〃	4.5	4.4	152.7	19.7	0.541

* Optical dencity of acetone extract of leaf blade (at the 663 mμ).

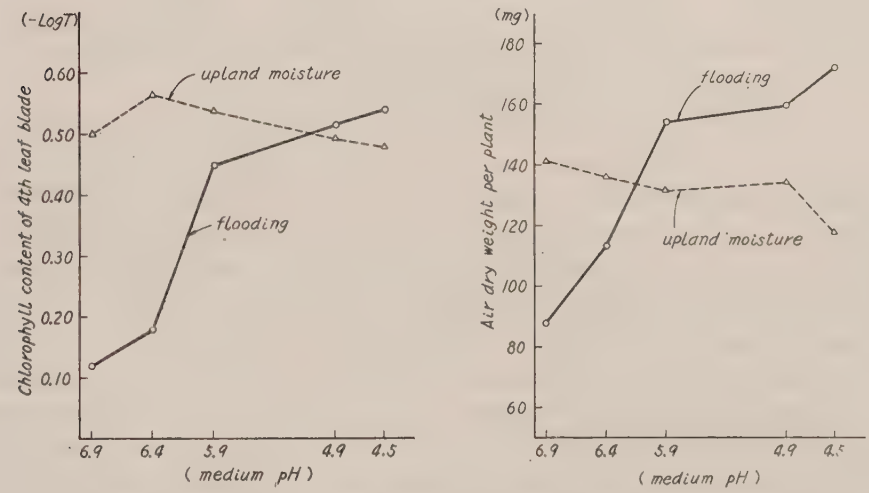

Fig. 1. Effect of pH and moisture content of synthetic soil media on the chlorophyll content and air dry weight of lowland rice *Nōrin-16*.

increasing to pH 4.5. In the upland series, on the other hand, it indicates the maximum value in the case of pH 6.4, a tendency toward gradual decrease observed as pH values grow lower.

The same tendency can be recognizable with air dry weight. What is noteworthy here, however, is the cases of relatively lower pH in the flooding series. That is to say, though the chlorophyll content of leaves in these treatments generally show lower values than those in the upland series, the air dry weight of the plants indicates higher ones than the highest one in the upland series. Such a difference in dry matter production may not be satisfactorily explained from the chlorophyll content, in other words, from whether the supply of iron is proper or not. Therefore, other factors effecting this phenomenon ought

to be expected. This problem will be refered to in Expt. 3 and discussion.

Experiment 3.

As the above experiments clarify, in the medium of synthetic soil, iron chlorosis takes place in rice especially under the water logging condition. It is also known on the other hand that the iron chlorosis in plants is accelerated by excess soil moisture and low temperatures.[25),26),31)] There are, however, some papers which report that the iron chlorosis of rice brought about under the calcaleous soil or wet soil condition, is removed by water logging.[7),26),27)]

Putting all these facts together, the following conjecture is possible. The increase in soil moisture it-self may behave as a factor to accelerate the iron chlorosis in plants. In natural soil with some quantity of organic matter, however, some conditions, such as poor aeration accompanied with the increase in moisture and high temperature, are supposed to make soil iron available secondarily. When the effect of iron supply thus caused acts more powerfully than that of moisture itself, water logging may probably produce the contrary effect of preventing the emergence of iron chlorosis.

For the clarification of such relationship, the effect of the medium moisture content and temperature on the emergence of iron chlorosis was examined by using two kinds of media, — one with organic matter and the other without it, — as mentioned in the following. Synthetic soil prepared in the same manner as in the experiments already described was used as the former, and paddy soil as the latter. The paddy soil was dark brown loam (pH 5.8) from the suburbs of Sendai city. 0.7 kg of the air dryed soil was mixed with 1.4 kg of unwashed river sand to make the texture of the medium resemble that of the synthetic soil as far as possible. Both the media were prepared by adding calcium hydroxide so that their pH values might be approximately 6.8, 220 mg of phosphorus used per pot. The method adopted was the same as that of Expt. 1 (A) except above. Lowland rice, var. *Nōrin 16* was seeded on Apr. 23rd, 1959, with 16 grains per pot. On 27th, after germination, the pots were divided into 3 groups, which were respectively placed under the following 3 temperature conditions.

(A) The first group was laid at high air and soil temperature in a heated green house.

(B) The second, in a tub in the same house, into and out of which the water from the aqueduct always flowed, high air and low soil temperature given.

(C) The last one, at a low air and soil temperature in a glass house.

On the same day, each of them was respectively under the following 4 levels of moisture conditions ; 60, 80, 100 and 140 % (water logging) of its full water holding capacity.

The harvest of each group was reaped; (A) on 15th, (B) on 19th and (C) on 25th of May. And the air dry weight of the top and roots and the chlorophyll content of leaf blades were measured. The results shown in Fig. 2, and the air and soil temperature during the experiment recorded in Table 5.

Table 5. Air and medium temperature in each group during the experiment.

Group		Max. temp. in a day			Min. temp. in a day			Aver. temp. during experiment
		max.	min.	aver.	max.	min.	aver.	
A	air	40	26	32.8	20	15	17.1	24.7
	medium	32	26	29.2	20	19	19.8	24.6
B	air*							
	medium	26	18	20.6	19	14	16.9	18.7
C	air	36	19	26.9	19	6	12.3	19.6
	medium	31	20	24.6	20	14	17.2	21.0

* Same as group A.

(note) A: green house with steam heating.
B: green house with steam heating, cooling around pot with running water.
C: glass house without heating.

According to Fig. 2, chlorophyll content generally exhibits a tendency to decrease with increasing moisture especially under the water logged condition. Such a tendency is less obvious in the paddy soil than in the synthetic soil. The tendency can not be observed in the case of the medium of paddy soil placed under (A), that is, under the condition of the high soil temperature. This phenomenon is supposed to be probably due to the iron made available as the medium reduction made progress. No effect of soil temperature can be discerned in the emergence of chlorosis in the flooded media of synthetic soil without any organic matter.

Next, the air dry weight of the plants increases in proprtion to the increase in moisture within the range from 60% to 100% of the water holding capacity, but decreases abruptly under the flooded condition. The decrease in the latter is due to the excess deficieny of chlorophyll content. And the increase in the fromer is a phenomenon in accordance with the result, obtained in Expt. 2, that within optimum pH range dry matter production is more in the flooding series than in the upland series.

That is to say, the chlorophyll content of leaf blades shows a tendency toward gradual decrease with the increasing soil moisture. This is an unfavourable factor for the dry matter production of plants. Nevertheless, air dry weight is apt to increase to the contrary. It is probably because the increase in the supply of moisture for rice plants acted as a favourable factor for dry matter production.

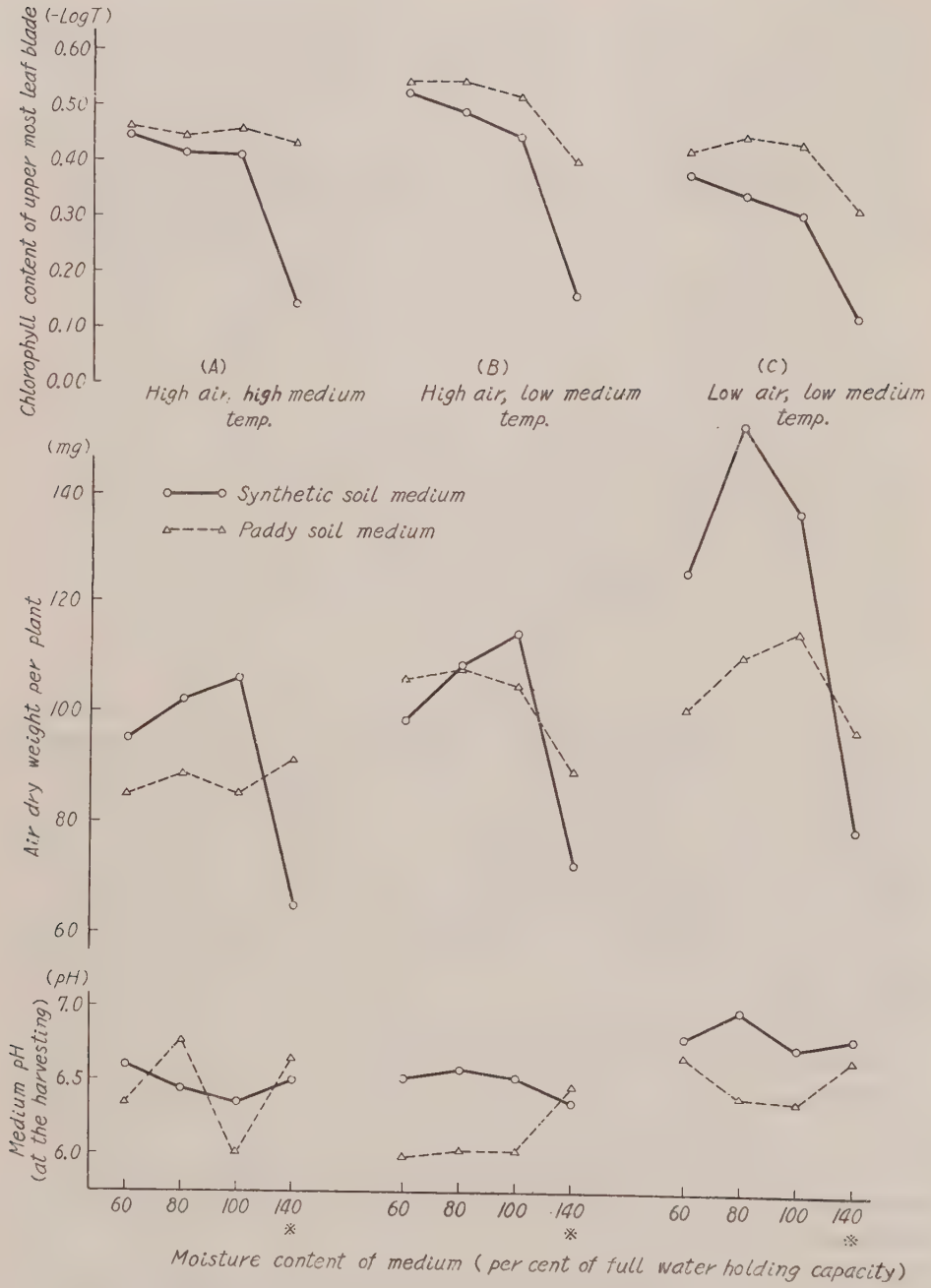

Fig. 2. Effect of medium moisture content and temperature condition on the chlorophyll content and air-dry weight of lowland rice *Nōrin-16*, especially on the difference in the effect between the synthetic soil and paddy soil media.
※ flooding condition.

In the case of the medium of paddy soil, however, no relationship above mentioned is generally observed. The reason is supposed to be that some change took place in the conditions of supplying nutrients in response to that in the moisture of the medium, which contained organic matter.

Experiment 4

Rice plants require iron to a high degree, and are susceptible of iron deficiency. Such a character of theirs is necessary for them to grow under the flooding soil condition with high concentration of ferrous iron, and it is thought to be one important side of the adaptability of rice to water logged soil. From such a point of view, is noteworthy the result of Expt. 1 that flooding induced chlorosis found in lowland rice, var, *Nōrin 16* was not discerned in barley var. *Aizu 2*.

As mentioned later, however, a phenomenon to be defined as flooding induced chlorosis was observed by Went[21),28)] in some upland crops. And some cases are also known in which the emergence of iron deficiency chlorosis is quite different in susceptibility between the varieties of the soybeans, to say nothing of between the plants of different species.[29),30)] For this reason, the followig experiment was made to obtain a general conclusion with respect to the difference in the susceptibility of flooding induced chlorosis between rice plants and upland crops.

Twelve varieties of rice, which are shown in Fig. 3, corn (var. *Wissconsin 606*), beans (var. *Tsuruari-oyako*), tomatoes (var. *Fukuju*) and radishes (var. *Kuroha-minowase*) were cultured in the synthetic soil in Aug. 1958. The medium pH were 6.2, 6.9 and 7.8 respectively. Seeding was carried out on July 31st or Aug. 1st. In the cases of the corn and radishes, 2 or 3 days after flooding was begun, chlorosis was clearly observed on the new leaves, in the media showing pH 6.9 and 7.8. The chlorosis, however, disappeared in a few days. In the tomatoes and beans, no chlorosis could be discerned both in the flooding and upland series. These 4 varieties of upland crops grew worse after the stage in the flooding than in the upland series, the beans and radishes showing especially poorer growth. Their roots also grew worse, exhibiting partial rot.

In the case of each variety of rice plants, on the contrary, chlorosis took place more clearly as leaf emergence progressed after flooding. The roots of the plants in the flooding series were generally shorter in length, and larger in number, and in addition, poorer in the development of their lateral roots than in the upland series, showing no symptom of root rot at all.

The harvest of the rice was gathered on Aug. 26th, and the chlorophyll content of upper-most leaf blades which finished development was determined, the results being shown in Fig. 3. The decrease in the chlorophyll content owing to flooding is essentially distinct throughout all the varieties used. Consequently,

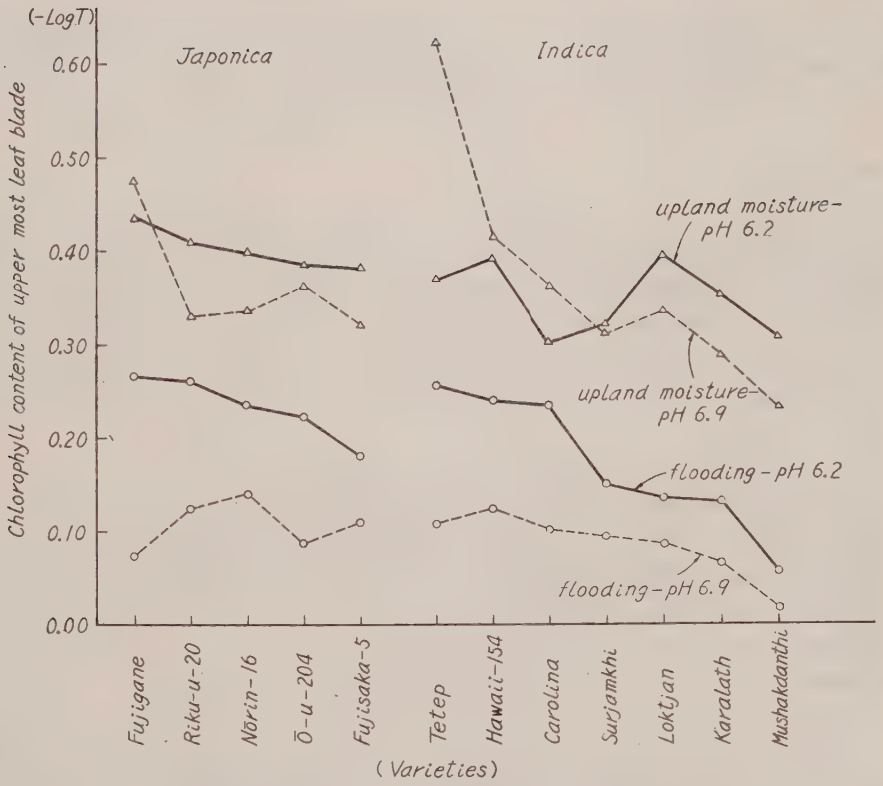

Fig. 3. Effect of pH and moisture content of synthetic soil medium on the chlorophyll content of the leaf-blades of rice, especially on the difference in the effect between the varieties.

it can be concluded that flooding induced chlorosis is a phenomenon which occurs commonly in each variety of rice plants.

A considerable difference in the degree of chlorosis emergence could be observed among these varieties. That is to say, most of the varieties used did not show chlorosis so clearly on the flooded media with pH 6.2. Even under the same condition, however, 4 varieties of rice, such as *Mushakdanti*, *Kalarath*, *Loktjan* and *Surjamkhi* showed conspicuous chlorosis, the phenomenon being the severest in the first variety. They are all the native varieties of Java or India, which are of indica type.

As mentioned above, in the case of upland crops, the symptom of flooding induced chlorosis did not appear, or was limited to a brief period directly after flooding in the present experiments. For the further confirmation of this fact, the following experiment was made on barley, var, *Aizu 2* and wheat, var. *Norin 55* in a cold climate (March 1959).

In the present experiment, the 4 levels of medium pH values were designed ;

6.5, 7.2, 8.1 and 8.7 and 310 mg of phosphorus used per pot. Besides, 0, 620, 1240 mg of phosphorus were added to the media of pH 6.5 and 8.1. After germination, they were divided into two groups, which were grown respectively in a glass house (average maximum day temp. : 19.6°C and average minimum night temp. : 6.5°C) and in a heated green house (aver. max. day temp. : 25.5°C. and aver. min. night temp. : 11.8°C.).

Both the barley and the wheat, — which was somewhat different from the result of Expt. 2 — showed chlorosis soon after flooding. The phenomenon, however, disappeared again in 5–10 days. Moreover, in those which were cultured in the green house, chlorosis occurred and disappeared earlier because of their faster leaf emergence velocity, showing a shorter period when the chlorosis could be observed. The case of no phosphorus application was an exception, that is, both the media of pH 6.5 and 8.1 presented the phosphorus deficiency symptom, exhibiting no chlorosis after flooding.

Putting together the results observed concerning the upland crops, the phenomenon of flooding induced chlorosis is likely to emerge in the case of upland crops. Under the condition in the present experiment, however, some factors effecting the removal of chlorosis are supposed to be brought into being succeeding to the chlorosis emergence. Accordingly, the problem as to what brings about such factors is one of those which are to be clarified in future.

IV. Discussion

The result of the present experiments is thought to mean that the susceptibility of rice plants to iron deficiency is furthered in response to the increase in moisture in the medium. The reason is that there can be no possibility that any chemical change is brought about in the medium by the increase in moisture because the synthetic soil used in these experiments contains no organic matter in it. And as mentioned in the experiment on the effect of planting density (Expt. 1–c), the change in the medium condition owing to the growth of plants can not either be thought to have cuased flooding induced chlorosis. From these facts it can be concluded that the cause of flooding induced chlorosis exists in a physiological change in plant.

As Expt. 2 clearly reveals, the phenomenon of flooding induced chlorosis is that the degree of chlorosis generally grows severer as the medium moisture increase. Conseqeuntly, this phenomenon is supposed to have some points in its mechanism of emergence at least partially common to the iron chlorosis furthered by the excess moisture of soil, which is observed in the upland crops in calcaleous areas[25),30–33)] and the upland rice in Japan.[26)]

According to Hass's report based on the research into the orchard soil in a calcaleous area, the soil under which iron chlorosis takes place shows a tendency toward the more increase in pH for the wet condition. Expt. 1 of the present

paper, however, made it impossible that the change in pH values in response to the increase in medium moisture is regarded as the cause of chlorosis. Thorne *et al.*[32] discussed on various factors in the causation of lime induced chlorosis, and concluded that, disturbing the metabolism of plants, high pH values, high lime, high soil moisture and low temperature cause the inactivation of iron in their tissues. Lindsay and Thorne[33] have lately attributed the progress of chlorosis owing to the excess moisture of soil to the excess accumulation of bicarbonate in soil, though the direct proof has not been afforded yet. And aeration is ineffective for the prevention of the iron chlorosis which appears in water culture, and from this it has been said that deficiency of soil oxygen caused by excess moisture is not the cause of chlorosis.[28),33),34)]

What is noteworthy concerning these problems is the observation of Went's.[25] Making an experiment by the technique of halved root systems and of lowering liquid surface, he recognized that tomatoes and cosmoses produced chlorosis, when thier root systems were completely submerged in a nutrient solution, and that the aeration made no effect on the recovery from it. He observed in addition that when new aerial roots were formed above the solution recovery from the chlorosis took place. Recently[21], it has also been reported that same chlorosis appeared when, nutrient solution was continuously sprayed in the root-fog chamber, where the roots were fully aerated. These phenomena of chlorosis observed by Went can, in no case, be thought to be due to the deficiency of oxygen or to the change of chemical condition in the medium accompanied with water logging, such as the accumulation of bicarbonate or available manganese. This supports the above mentioned conclusion of the author's that the cause of flooding induced chlorosis is the change in the physiological function of plant brought about in response to the increase in the medium moisture itself.

If the above conclusion is tentatively admitted here, it will be unnecessary to mention that the same mechanism as above partakes, as its major cause, in the emergence of iron chlorosis in the natural soil of excess moisture. And such chlorosis furthered by low temperature is supposed to be chiefly due to the depression of microbial activity, which is expected to contribute to the availabity of iron. Its evidence can be mentioned here, that is, no effect of temperature on the degree of flooding induced chlorosis could be recognized in the synthetic soil media (Expt. 3).

It is commonly admitted that rice plants are poorer in the capacity of utilizing iron than barley and wheat, and consequently that the security of iron supply specially means much as medium conditions required by rice in their water culture. For example, the optimum pH of the culture solution is lower in rice than in barley and wheat, and ammonium nitrogen and comparatively low phosphorus concentration are generally regarded as favourable.

With respect to the case where synthetic soil is used for a medium, under

the flooded conditions, there is a tendency quite similar to the case of rice plants water culture. That is to say, the optimum pH of the medium for the growth of rice is comparatively low (Expt. 2), and the phosphorus deficiency symptom hardly appears, while iron chlorosis emerges quite easily owing to the excess phosphorus (Expt. 1). Under the upland moisture conditions, on the contrary, rice plants seldom show iron deficiency, but suffer from phosphorus deficiency instead, and the optimum pH of the medium is comparatively high. Viewed from these respects, there is a tendency nearer to that which barley exhibits when water cultured.[22–24] These facts suggest that under the upland moisture condition, rice plants come to require the nutritional condition similar to those of upland crops like barley, compared with the case of water logging.

It may be explained as follows that the nutritional character of rice show such a change. Barley has a fixed character corresponding to the upland moisture condition, while rice are supposed to have one suitable for water logged condition. And such a character may be adaptable to the different moisture condition within a certain limit. Rice plants probably have a capacity to adapt themselves, to some degree, to the physiological state which is especially concerned with water relation, responding to the upland moisture condition, but can not enlarge the capacity to the same degree as that of upland crops. The difference in susceptibility of iron deficiency chlorosis, observed among various plants and in different moisture environments, can be supposed to reflect such a character.

It helps one understand that the chlorosis brought to plants by the excess moisture of soil and rice-plants' keen susceptibility to iron deficiency are both essentially governed by the same mechanism. And the nutritional character of upland nursery rice seedling refered to in the introduction, is also suggested in the reflection of the physiological state of water which they acquired in the upland moisture environment.

Furthermore, based on the hypothesis is possible an expectation that the most fundamental favourableness for growth of rice in submerged soil is the supply of moisture, and that the nutritional advantage is only a secondary element. In Expt. 2, the dry matter production of rice was higher in the flooding series than in the upland series in the media with optimum pH range. In Expt. 3, it increased in proportion to the increase in the medium moisture contrary to the decrease in the chlorophyll content. These results can be regarded as the experimental facts which prove the above expectation. Further examination, however, will be necessary in future to gain a satisfactory conclusion about this problem.

The author are indebted to Mr. Hideo Okajima and Norindo Takahashi for valuable advice and discussion in the course of the work.

V. Summary

The growth response of rice plants to the change in the medium moisture was examined, with synthetic soil as its medium, for the purpose of clarifying a problem as to what change the moisture environment brings to the inorganic nutrients required by rice. As a result, it was found that they are keenly susceptible of iron deficiency chlorosis under the flooding than under the upland moisture condition. Therefore, for the cause of the chlorosis, further examination was carried out :

1) of the effect of medium pH, planting density, the phosphorus levels of the medium, and the application of chelated iron to the medium and to the leaves, on the chlorophyll content and dry matter production ; especially on the difference in the effect between the upland and flooding moisture conditions.
2) of the effect of the quantity of the medium moisture and soil and air temperature on the chlorophyll content and dry matter production of rice, especially on the difference in the effect between the media with or without organic matter.
3) Of the difference in the susceptibility to flooding induced chlorosis among various plant species and among the rice varieties.

The consideration based on the above experiments are as follows :

1 The cause of flooding induced chlorosis rice plants show is supposed to consist in the decline of their capacity of utilizing iron owing to the change of the physiological condition of their bodies brought about by the increase in the medium moisture itself.

2 Based on the above conjecture, the excessively high susceptibility of the rice to iron deficiency and the progress of iron chlorosis owing to the excess moisture of soil, — these two facts can be reasoned without contradiction.

3 From the viewpoint of inorganic nutrition, the upland nursery seedlings of rice show a special character different from that of lowland ones. The reason is surmised to be partly due to the fact that the physiological water relation in the former tissues is different from that in the latter ones.

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Studies on the *Helminthosporium* Leaf Blight of Rice Plant*

Part 3. Some histological observation on the inner epidermal tissues of leaf sheaths inoculated with the blight fungus.

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I. Introduction

In the previous paper[6] the authors reported that the blight lesions developed in the inner epidermal tissue of leaf sheath by artificial inoculation could be divided into five types from the patho-histological point of view. Distinction was based on the comparative observation on the pathological pictures of the infected tissues established after about two days' incubation. But, as it was shown elsewhere[6], a cellular alteration of the affected host-cell can be confirmed microscopically within a few hours after inoculation. Then it would be necessary to follow the trend of pathological change from fungal penetration to establishment of each lesion-type. The results of the observation on the developing processes of lesions belonging to the types I and II will be given in this paper.

The authors wish to express their appreciation to the members of the research group of their Institute engaged in the "Studies on muck paddy fields" for their assistance and many helpful suggestions.

II. Materials and methods

Leaf-sheaths used in the inoculation tests were obtained from the ninth (9/0) to the thirteenth (13/0) leaves on the main haulum of rice plants (*Norin No. 16* variety). Cultivation of the test plants, preparation of the inoculum and the inoculation procedure were the same as those described in the previous paper (9), but the concentration of spore suspension was more diluted than that in an ordinary sheath inoculation to obviate a possible mutual influence among developing lesions. Inoculated sheaths were incubated in a moist chamber at 24°–28°C.

After certain hours' incubation the infected inner epidermal tissues were stripped in a few cell-layers' thickness with a razor, and examined under the microscope at definite intervals. On mounting them thin glass capillaries were

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inserted between the cover slip and the slide glass to prevent the injurious squeezing by the cover slip. During the microscopical observation they were sometimes supplied with fresh water.

Such living epidermal strips are not appropriate for a long-time observation because of the progressing autolysis[5]. So in case of the prolonged observation the epidermal strips which were removed from the incubated sheaths successively at certain intervals were preserved in 20% alcohol solution and examined microscopically.

The death of the affected host-cells and the intracellular hyphae in infection process was examined by means of the vital staining method with neutral red (0.01 %) or of the plasmolyzing method with hypertonic sugar solution (0.5–0.8 M). Sometimes *Murasaki-ine* variety, which contains anthocyan pigment in its epidermal cells and produces the spreading type of lesion (types II and III), was used to judge life or death of the host-cells. Death may easily be recognized by the disappearance of their red color.

III. Results of the observation

1. Behavior of the blight fungus before invasion

Germination: Nishikado[4] reported that the blight conidiospores floating on distilled water can slightly germinate within an hour at 20°C. As shown in table 1, with the blight strain used in this study, about half of the spores under test germinated for 3 hours at 24°C; and it was the same also on the inner epidermal surface of leaf-sheath.

Table 1. Germination of the conidiospore of *C. miyabeanus* floated on distilled water at 24°C.

Incubation hours	Number of spores, observed	Percentage of germinated spores	Number of germ tubes per a spore (aver.)
1.5	240	6.3	1.0
2.0	300	12.8	1.5
3.0	275	48.8	2.3
4.0	196	89.0	3.0
5.0	177	82.5	2.8

Formation of appressorium: Usually one conidiospore emerges on one or more germ-tubes; and the appressoria are produced at their respective ends. As shown in table 2, germ-tubes emerged mostly from both the ends of a spore. Consequently infections may often be secured through almost simultaneous penetrations by some appressoria produced by one and the same conidiospore.

Simultaneous penetrations accomplished by some germ tubes from one spore would bring difficulties to follow microscopically the infection process.

In general, the tip of the germ tube swells slightly to form an appressorium and the spore-cell content moves into it. Though vegetative hyphae may develop

Table 2. Showing the position of the blight conidia, from which germ tube emerged in distilled water (after 4 hours' incubation at 24°C).

Number of spores, observed	Percentages of the germ tube from					
	Basal or apical cell (1)*	Both basal & apical cells (2)	Middle cell (1)	Apical or basal, & middle cells (2)	Terminal & middle cells (3)	Other cells (2)
239	18.0	44.6	3.3	15.8	10.9	7.5

* Number in the parenthesis indicates those of germ tubes.

all over the epidermal surface, neither appressoria nor affected host-cells are found. It often happens that the swelling and the wall-thickening occur at an intermediate cell of aerial hypha, from which hypha may further grow. In a few cases an appressorium-like body is formed. As shown in table 3, three-fourths of the germinated spores formed appressoria after 3 hours' incubation at 24°C. Most of the appressoria were formed on the joint part of host-cell walls (table 4). Goto[2] also reported the similar observation.

Table 3. Germination of conidiospores and formation of appressoria on the inner epidermal surface of leaf-sheath (Aug. 2, 1956). Sheaths were obtained from the 10/0 leaves (*Norin No. 16* variety). Inoculated sheaths were incubated in a dark room at 24°C.

Incubation hours	Number of conidia, observed	Percent. of germinated conidia (%)	Percent. of conidia, formed appres. (%)	Percent. of appres., penetrated (%)	Number of appr., formed by one spore (aver.)	Number of cells invaded by an appr. (aver.)	Discoloration of host cell
1	235	7.5	?	—	—	0	
3	164	61.4	75.6	12.5	2.1	1.0	±
5	138	93.8	90.2	33.2	2.5	1.0	+

Table 4. Showing the part, on which the appressoria were produced on inner epidermal surface. Leaf-sheaths were obtained from the 10/0 leaves. *Norin No. 16* variety. July 31, 1956.

	Number of appressoria, observed	Percentage of the appressoria settled on		
		Joint part of cell walls	Peripheral part of cell	Other parts
		%	%	%
Control plant	218	65.2	23.5	11.3
Additional N plant	194	61.7	29.0	9.4

2. Invading process of the blight fungus and the pathological changes of host-cells.

Observation on the type I lesion

The incipient anomaly of the host cells was observed immediately after

transference of spore-cell content into the swelled tip of the germ tube. After settling of the appressorium on the epidermal cells, cell walls began to swell and their separation occurred (the formation of infection cavity). (Pl. I, fig.F). At this time fine discolored granules appeared near the appressorium, and the cell wall under it turned to yellowish brown color. Nucleus also swelled and migrated toward the appressorium. Browning went on rapidly with the formation of infection cavity.

Then granulation of cytoplasm occurred before long; light yellowish fine granules appeared along the discolored cell-wall or around the swelled nucleus. Their Brownian movement was further intensified. Thus the degeneration of the infected cells went on rapidly. And finally they were uniformly filled with yellowish brown granules.

Pathological changes above mentioned could be observed within 3–5 hours after settling of the appressorium. But browning of the infected cells remained even after 10–15 hours. The invaded hyphae showed no growth within such degenerated host-cells. However conidiospore and appressorium on the epidermal cells could still be stained with neutral red. Infected cells might lose both their plasmolyzability and stainability at about the time of discoloration of the nucleus.

The host cells adjacent to the infected did not show any other conspicuous alterations than the fact that their nuclei swelled slightly and that moved toward the infected cells. However, when the tissue was soaked in hypertonic sugar solution (0.6–0.8 M), the affected cells indicated ceratin abnormalities as shown in plate I (fig. A–D). Healthy cells took the convex form of plasmolysis, while the affected did concave or irregular one. Furthermore, when the plasmolyzed cells were transferred to distilled water by successive passing through the sugar solutions with decreasing concentration, the host cells adjacent to the infected often collapsed in the course of deplasmolysis. Consequently it is presumable that the pathological cellular alteration might already take place even in the uninfected cells with healthy appearance around the lesion. An example of the pathological alteration in the developing process of the type I lesion was represented schematically in plate II (fig. 1).

Observation on the type II lesion.

As shown in elsewhere[6], on the epidermal tissues, where the occurrence of this type of lesion predominates, other types may also appear. It was difficult, in such cases, to follow successively the trend of pathological alterations of the same cell or cell group in an inoculated epidermal strip especially at the earlier stage of infection. The following description was based on the results of observations with different strips taken successively at definite intervals after inoculation.

The process of cellular alteration of this type from the settling of appressorium

to the formation of infection cavity seemed to be almost similar to that of the former type. Slight discoloration of the cell content occurred prior to the penetration of hypha into the host cell. Granules were somewhat coarse. Granulation of cytoplasm around the appressorium or along the cell wall and browning of the cell content did not proceed so rapidly as in the former. Degeneration of the host cells within a lesion did not proceed uniformly. Many uninfected cells surrounding the infected cell-group degenerated and turned brown rather rapildy. Of course, no plasmolysis and vital staining occurred in them.

During the course of such cellular alteration the intracellular hyphae ceased to grow and were enveloped within the infected cell group. But some of them could succeed in growing further into the surrounding uninfected cells. Sooner or later the intracellular hyphae took a chain-like form consisting of the thick-walled, mycelial cells. They protruded conidiophores, on which conidiospores might be formed. An example of the pathological alteration in the developing process of the type II lesion was shown schematically in plate II (fig. 2).

As above mentioned, observation on the developing process of this type of lesion (II) was made through not successively, on the same living strip. Survival of the host cells in the earlier infection stage could not clearly be confirmed. However its pathological change was readily distinguishable from that of the former type (I) by the appearance of extensive discolored area around the infection cavity in about 6 hours after inoculation. The discoloration and granulation in the invaded cells were far less intensive than those in the former. Their nuclei usually turned pale brown. In these cells both the occurrences of plasmolysis and vital staining were not observed. In addition it was observed that in the epidermal tissue of *Murasaki-ine* infected by the pathogen its red color disappeared in advance of the invading hyphae. Considering these observations, it was presumed that the coexistence of the host cell and the pathogen in the infection court might be restricted only for a short time even in the early infection stage, and the death of the latter preceded that of the former.

From the results of the above observations, the cellular alterations and the fungal behavior in the developing processes of both types of lesions are summarized as follows :

Type I : (i) In the early infection stage, Brownian movement of pale brown granules in the affected cells around the appressorium ceases rapidly, and coagulation of cell contents soon arises ; and further development of the primary intracellular hyphae is hardly visible. Consequently it can be assumed that the intracelullar hyphae may stand in a circumstance of being incapable of further development in the earlier stage of infection.

(ii) Conidiospore and appressorium on these degenerated cells are still alive even after the death of the host-cells.

(iii) The neighboring cells around the infected cells also show some

abnormalities ; that is, their nuclei swell slightly and migrate toward the infection cavity. It appears, however, that they are still alive even in about 40 hours after inoculation. Survival of the neighboring cells seems to be closely related to the rapid necrosis of the invaded ones. Thus, it would be a hypersensitive reaction.

Type II : (iv) In the early stage of infection, pale brown discoloration occurs in an extensive area surrounding the infection cavity, but discoloration of granules and coagulation of cell content in the infected cells are far less intensive than those in the former type (I). The secondary intracellular hyphae grow well into the adjacent cells, in which granules show still intensive Brownian movement.

(v) In the later stage the intracellular hyphae developed in the infected area which had been enveloped by the necrotic cells cease to grow ; and some of them die within about 40 hours, whereas the survived hyphae often turn pale brown, thick-walled mycelia, and in about 3 days conidiospores are produced from them.

The above mentioned observations were made exclusively on the developing processes of two types of lesions, where discoloration occurred in the early infection stage, and those of the other types of lesions, where discoloration could be hardly seen microscopically remained to be investigated. In order to make clear the defence reaction of host cells against the blight infection, it is necessary to make comparative investigation of each type of lesion as shown in the infection process of *Phytophthora infestans* by K. O. Müller *et al.*[3] and Tomiyama recently.[7),8),9)]

IV. Summary

Cellular alterations of the host and behavior of the invading pathogen in the blight-inoculated tissue of inner epidermis of leaf sheath were investigated microscopically with special reference to the developing processes of lesions belonging to types I and II.

1. The blight spore settled on the epidermal surface germinates after 3 hours' incubation at 24°C and forms appressoria usually on the joint part of cell-walls. Infection hypha having emerged from the underpart of appressorium finds its way in middle lamella (formation of infection cavity) and invades into the host-cell.

The nuclei of the host cells tend to migrate toward the infected cell or the invaded hypha. The infected cells turn to discolor and begin to granulate.

2. Type I : In the early infection stage the infected cells show a rapid necrotic reaction. Small aggregates of necrotic brown cells are usually found in about 5 hours after inoculation. But they do not further enlarge. Host cells around the necrotic cells seem to be still alive even after about 40 hours. Conidiospores and appressoria settled on the necrotic cells remain to live.

3. Type II : Pathological change of this type is readily distinguishable from that of the type I in about 5 hours after inoculation. Necrotic reaction of host cells as seen in the type I is not observed at the early stage of infection, but

slight discoloration of the infected cells may be seen before complete invasion. Development of the secondary intracellular hyphae into neighboring cells is well observed. With gradual darkening of the necrotic cells their development seems to be hemmed. Then they often turn into the brown thick-walled mycelia, from which conidiospores may be sometimes formed within 3 days' incubation.

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Explanation of plates

Plate I. Photomicrograph of the inner epidermis of leaf sheath (*Norin No. 16* variety) inoculated with the blight fungus (incubation temperature was about 24°C). S : conidiospore ; g : germ tube ; a : appressorium; h : intracellular hypha ; n : nucleus of host cell ; c : infection cavity. A-B. Type I lesion developed for 10 hours after inoculation: non-plasmolyzed (A) and plasmolyzed host cells in 0.8 M sugar solution (B). C. Plasmolysis of the healthy host-cells incubated for 18 hours. D. Plasmolysis of the infected and its adjacent host cells after 20 hours' incubation (type I lesion). E. Cellular alteration after 4 hours' incubation. The nuclei of host-cells around the appressorium swelled and migrated toward the infection cavity. F. Infection cavity (3 hours after inoculation).

Plate I

Plate II

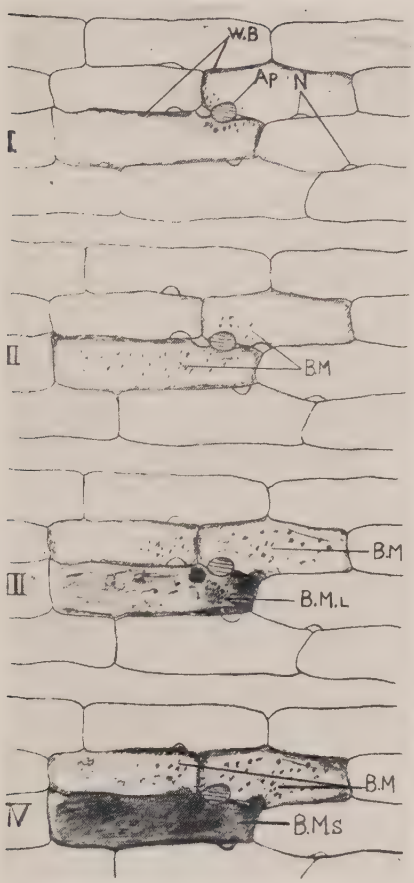

Fig. 1

Fig. 2

Plate II. Schematic representation of the pathological alteration of the blight-infected inner epidermal cells.
Ap : appressorium ; N : nucleus ; W.B. : Discoloration of cell wall and granulation; B.M. : granules, showing an intense Brownian movement ; B.M.L. : granules, showing a weak Brownian movement ; B.M.S. : granules, standing still; S : conidiospore ; T : conidiophore ; H : chain-like mycelia with thick wall.
Fig. 1. : Type I. I : 4 hours after inoculation ; II : 4 2/3 hours ; III : 6 hours ; IV : 7 5/6 hours.
Fig. 2 : Type II. A : 24 hours after inoculation ; B : 36 hours ; C : 74 hours.

Temperatures during Grassland Fires and their Effect on Some Species in Kawatabi, Miyagi Prefecture*

Mitsuya Ito** and Shigeru Izumi
(Received on February 23, 1960)

I. Introduction

In the semi-natural grassland of north-eastern parts of Japan, fires are frequent and periodically reburn the same areas. Therefore, a few general conclusions had been drawn previously concerning the effect of fire on the grassland in our country.[1] However, there is no evidence in their literature of our country that temperatures produced during fires play any significant role in resprouting of some bush species.

In the following our studies, attention was focused on the shrubs which make up the bulk of the vegetation of grassland burns.

II. Outline of the investigated land

The place subject to the present investigation was the semi-natural grassland at the Kawatabi Farm attached to the Agriculture Department of Tohoku University. The grassland is located 300–600 m above the sea-level, around which there are seen oak forest predominantly. As reported summarily on vegetation and soils of the grassland[2][3], the plant community of the land is dominated by tall grasses often 1 to 2 m high and belonging to bunch forms. The major dominant is *Miscanthus sinensis* which is associated with herbs such as *Lysimachia clethroides*, *Potentilla Freyniana* and *Astilbe congesta*. The bush species, most of which resprout following fire, are found with the codominant, in their region. Such shrubs and young trees were *Weigela hortensis*, *Salix vulpina*, *Lespedeza bicolor*, *Hydrangea paniculata*, *Quercus serrata* and *Eubotryoides Grayana* etc.

The land is volcanic ash soil characterized with a thick humus layer. There have been carried out grass-cutting in the autumn and burning for many years. When cutting and burning are stopped, such young trees and shrubs invade the grassland which trend to develop into a wood. Therefore, the vegetation of this land is the representatives maintained by cutting with an intermittent firing.

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III. Air temperatures and heat penetration of soils during grassland fires

It may be sure that the abundant appearance of these bush species on this land is owing to a direct relation between resprouting behaviour and fire. The fact that fire acts as a stimulus to resprouting of some plant species is well known.[4)]

In order to test this acts of fire it was necessary to consider the upper limits of air temperature during fires in the greassland and the penetration of heat at various levels near the soil surface where most root-crowns occur.

On the field investigations of the grassland, maximum thermometers were placed on the soil surface and at 2, 5, 15, 20, 30 cm above the surface, respectively. Moreover, there were burried at 1, 2, 5 cm below the surface, respectively. Direct readings were made from the thermometers and the temperatures recorded after the plant meterials was burned.

On the other hand, in order to estimate the probable maximal temperatures during fire, the equivalent (400 g as dead grass) of the same grassland vegetation (excluding shrubs) from a quadrat of 1 m² was built into a pile and lit. Such a fire will represent the probable maximal intensity, assuming that the whole of the vegetation is burned. This experiment was made in the experimental farm of Sendai on February 26.

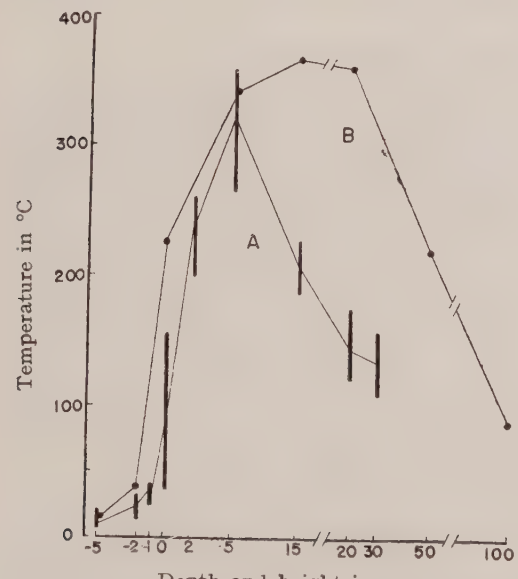

Fig. 1. Air temperature at various heights and heat penetration of soil at various depths during fires. Curve A : in the field, Curve B : in the experimental farm.

The results obtained are summarized graphically in Curve A and B, Fig. 1., respectively.

The surface temperature varies from 55 to 230°C. At a depth of 2 cm the temperature was 38°C, while at 5 cm a temperature of 15°C was recorded. The air temperature at the heights of 20, 30 and 50 cm exceeded 100°C, respectively. At a height of 1 m the air temperature was 92°C. Particularly, at the heights 2, 5 and 15 cm exceeded 200°C, respectively. Moreover, this figure show that the temperatures measured at the heights of 15 and 20 cm above the surface in the experimental tests were considerably higher than those produced during fires in the investigated field of Kawatabi.

This reasoning is based on the fact that grassland fires rapidly pass over the soil surface as they consume the plant materials.

From these results, it can be seen that the soil depth below the 2 cm level, the temperature was not sufficiently high to harm any underground organs. Therefore, the depth below the surface of the root-crown of shrubs will be of great importance in its survival.

IV. Resprouting of some bush species

In spite of such burned conditions, it is characteristic of our grassland that after fire regeneration of shrubs in common and dense cover of shoots appear from root-crown which has tolerated the fire. Therefore, in August the resprouting of some bush species (viz. *Weigela hortensis*, *Salix vnlpina*) was observed following fires of April in our grassland.

Data was compared the burned-over district with adjacent unburned district (Table 1).

Table 1. Comparative plant growth of bush species in burned and unburned areas.

Bush species	Burned			Unburned			Depth of root-crowns surface (cm).
	(1) Number of stocks per 1 sq. m.	(2) Number of shoots per 1 sq. m.	(3) Minimal heights of resprouting bud (cm).	(1)	(2)	(3)	
Weigela hortensis	1.05	4.2	0–4	0.25	0.9	32<	1–3
Salix vulpina	1.03	7.6	0–5	0.35	1.5	28<	2–3

As a result of these observations it was noted that new bush shoots numerously appeared on the burned site than the unburned site. Moreover, the root crowns of bush species are burried deeply to avoid high temperatures. The fact suggests that the soil acts as an effective insulator against heat penetration during fires. Thus, it has become clear that fire is an important factor to persistence of certain bush species in our semi-natural grassland.

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Photo. 1. A general view of burned-over grassland at Kawatabi, after two weeks from fire. *Miscanthus sinensis* is dominant in this area.

(1)

(2)

Photo. 2. Close-up view of representative bush species in burned area. (1) *Weigela hortensis*, (2) *Salix vulpina*.

A New Butanol Producing Anaerobic Nitorogen Fixer, *Clostridium aurantiacum* nvo. spc.

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(Received on March 1, 1960)

I. Introduction

In the summer of 1956 and 1957 the authors made the semi-quantitative investigations on the transition of anaerobic nitrogen fixing batcteria of *Clostridium pasterianum* type in the rice planting pot soils by the dilution method. During the course of those investigations the authors were encountered with a bacterium which produced orange-red sediments, that is, in the pot soil in the fixed temperature room at 27°C at the end of July, 1956 and in the pot soil at 23°C at the begininng of September, respectively. In other cases which investigated the soils sampled other than the aforementioned periods from the glass room at 17°, 23°, and 27°C, and the natural temperature, the authrs could not obtain any strains characterized by such a colorization in all appearances. Each strain obtained in 1956 and 1957, though having undergone fermentation, showed relatively long the lag time for the initiation of growth and in its culures bore orange-red colored heads and sediments which were changed into brown color with the lapse of time. Since the strains could be transfered ten times in the Starkey medium not containing other extragenous nitrogen compounds than nitrogen atmosphere, those are evidently nitrogen fixing bacteria. As they accumulated a considerable amount of butanol as fermentation products, their morphological and physiological characters were precisely investigated. The results obtained are reported in this paper.

II. Method

Media : (i) Starkey's medium[1] : Glucose 20 gr., K_2HPO_4 0.5 gr., KH_2PO_4 0.2 gr., $MgSO_4 \cdot 7H_2O$ 0.2 gr., NaCl 0.2 gr., $CaCl_2 \cdot 2H_2O$ 0.05 gr., $FeSO_4 \cdot 7H_2O$ 0.025 gr., Na_2MoO_4 0.005 gr., $NaWO_4$ 0.0005 gr., $MnSO_4$ 0.0005 gr., H_2O 1l., pH 7.0.

(ii) Medium for the use of *Cl. pasteurianum*[2] : Glucose 20 gr., K_2HPO_4 0.8 gr., $MgSO_4 \cdot 7H_2O$ 0.2 gr., NaCl·$FeSO_4 \cdot 7H_2O$, $MnSO_4$ traces., tap water 1l : pH 7.0.

(iii) Davies' medium[3] : Glucose 20 gr., peptone 0.25 gr., KH_2PO_4 0.5 gr.,

The 225th report of the Institute for Agricultural Research, Tohoku University.

K_2HPO_4 0.5 gr., $MgSO_4 \cdot 7H_2O$ 0.01 gr., $FeSO_4 \cdot 7H_2O$ 0.01 gr., asparagine 1.0 gr., H_2O 1l : pH 7.0.

(iv) Corn medium : Crashed corn 50 gr., tap water 1l.

Media a, b, and c were pasteurized for 15 minutes under the pressure of 15 lbs., and medium d was pasteurized for 30 minutes under the pressure of 30 lbs.

Other media which have special compositions are described in the paragraph of the experimental results.

Method of culture : The culture using test tube was usually employed except large-scale cultivation. The test tube had a rubber stopcock which was attached to a glass tube, equipped with the Hoffmann's pinch-cock in order that the reduction of pressure and the substitution of purified nitrogen gas may be possible.[4] The culture medium which was poured into the test tube at an amount of five to ten ml was sterilized, to which the solution of subculture were inoculated and cultured on standing at 30°–37°C after deareation or the substitution of purified nitrogen gas.

III. Results

1. Isolation of bacterial strains :

The soil in the rhizosphere of submerged pot transplanted with rice plants at either the ordinary or constant room temperature was sucked up with a sterilized wide-mouth injection tube. Five ml of the sucked soil was suspended homogeneously in 45ml of sterilized tap water and subjected to the serial dilution treatment up to the extent of 10^8 fold. 1 ml of each diluted solution was inoculated into 5 ml of Starkey's medium and cultured at 30°C under nitrogen gas atmosphere. From the submerged pot soils in both 27° and 23°C fixed temperature rooms, their 10^3 and 10^4 fold diluted solutions gave rise to orange colored sediments at the end of July, 1956 and at the beginning of September, 1957, respectively. The supernatant fluid of the incubated soil solution was colored in orange-yellow and the color of the cultured medium was changed into brown with the lapse of time. However, other cultures obtained except those seasons were uncolored.

The bacterial strain obtained in 1956 could be preserved unitl August in 1957 by the repeated transfer at intervals of one to two months using the Starkey's medium, but the bacterial growth was entirely abolished. Since the organism could survive in over ten fold transfers in a medium lacking any of extragenous nitrogen compounds, it is evident that the organism present in each transfer was capable of fixing nitrogen gas. Analysis of the products which was made in March, 1957 obtained the results shown in Table 1.

As shown in Table 1, the fermentation products by the bacteria were consisted chiefly of butanol and ethanol, while acetone and volatile fatty acids were of negligible amount. The typical clostridial cells were well detectable by microscopic investigation. The organism obtained in 1957 was also studied and the same

Table 1. Fermentation products of a new butanol producing anaerobic nitrogen fixer. (mg/ml.)

Medium.	Glucose		pH	Acetone	Butanol	Ethanol	Butyric	Acetic
	Added	Consumed						
Davies	21.5	2.6	5.6	0.17	5.27	1.52	0.03	0.08
Davies with liver piece	20.0	1.7	4.6	0.1	5.24	1.41	0.41	0.09
Davies (with $CaCO_3$)	20.0			0.1	6.45	2.25	0.6	0.13

Analytical method
Glucose : Somogyi method, Acetone : microdiffision method
Alcohols : Johnson method, Acids : distilation method

results as above described were obtained. Then, the results described hereafter were all performed by the organism obtained in 1957.

This bacterial strain had extremely long lag period in the Starkey's medium, usually for 4–5 days and sometimes for more than twenty days. However, after the initiation of growth, its development finished completely within a relatively short time, i.e., 48-72 hours. The addition of a nitrogen compounds like peptone to the medium shortened the lag period considerably. As the transferring of peptone growing cells into the non-nitorgen medium still gave rise to the growth of the bacteria. The Starkey's medium containing 0.3 per cent of peptone was used for purification, where the bacteria were cultured by the use of a modified Weinberg tube[4] or an anaerobic plate. Colony formation of the organism was well attainable by this means, and the strain thus purely isolated was investigated on its morphological and physiological properties.

2. Microscopic observation :

The bacterial cells cultured at 30°C in the Starkey's medium containing peptone were microscopically observed.

The younger cells within twenty four hours culture were round edged rod, being $1\sim2\times5\sim6\ \mu$ in size. The cells were actively motile and usually singular and sometimes in pairs in the form of chains of two or more (fig. 1). There appeared cells converted into a spindle shape in the ripe culutre. At first, granules were well discernible at the terminal of the cells stained with crystal violet, while later the formation of spores were observable (fig. 2). The spores were of oval form in the size of $1.5\times2.0\ \mu$ and localized at the terminal or subterminal. These spores were stable at 100°C for a minute.

Staining the cells by the Nishizawa and Sugawara's method,[5] these were recognized peripherally flagellated. Gram staining was positive. The cells were stained in purple-brown color with iodine, so that they were hardly recognized Granulose-positive,

3. Growth on various media :

(i) Starkey's medium : As described earlier, the bacteria which grew in the atmosphere of the molecular nitrogen without any nitrogen source showed orange-red coloration at the bottom of the test tube after a considerably long lag period, and then both viscous matters colored orange-red and bubbles floated on the upper part of the test tube. A viscous layer was also formed at the height of about one fifth of the test tube from its bottom and the middle layer was somewhat tubrid and bubbled with shaking. After the completion of growth, the middle layer became clear and showed no bubble, but there were no great changes in all appearances. When the cock attached to the tube was opened, there occurred a strong smell of butanol. These characters were common to those obtained from the cultures in the following media.

(ii) Starkey's medium containing peptone : In this medium the lag period was remarkably shortened, and there took place such an intensive fermentation that orange-red viscous matters containing bubbles floated up and were accumulated also at the lower part of the test tube. The liquor was colored lightly with orange-red and changed into brown with the lapse of time.

The surface colonies on the agar plate were of a tabular form whose surface and periphery both were smooth. The colonies inserted into agar medium were of a round or oval form and were all colored with yellowish orange color.

(iii) Davies' medium : Cultural featured of the organism grown in this medium showed no apparent differences from those in the Starkey's medium. The silky turbidity showing the bacterial growth occurred in first five to six times transfers in this medium and then the organism stopped if not at all to grow. In this case, there were observed similar cells to the above mentioned by microscopic observation but those were hardly stained with crystal violet and inside of the cells were recognised to contain relatively stainable granules. This was especially the case when the organism was cultured at 37°C in this Davies medium, containing peptone as well as asparagine as nitrogen sources.

(iv) Bouillon broth medium : Growth of the organism was not observed in this medium under any circumstances.

(v) Corn medium : The active fermentation took place within 24 hours and finished in 72 hours or so. The culture became colored fine yellowish. The successive transfers in this medium gradually weakened the color production and decreased the fermentative activity. When the cells of repeated transfer in this medium were again transfered into the Starkey's medium containing peptone, there was obtained a strain which produces white viscous liquid. This strain was isolated from white colored colony formed by means of a modified Weinberg tube method described above. The morphological and other characters of this strain are closely resembled to those of the original one. The orange-red pigment producing strain was temporarily named strain 0, while the colorless organism

strain 0-1. The latter white strain was sometimes easily changed into the former yellow pigment producer by the transfers into the potato slant and then it still remains to be studied whether the white-colored strain would be a temporal variety of the yellow-colored strain, or a blended strain of the two strains different in pigment production and almost analogous in other characters would cause such a difference on occasions.

(vi) Potato slant[5] : The growth of the organism spreaded widly on the potato medium and the culture in this case became organge-red colored vividly and the colony surface was neither viscous nor rising. The condensed water at the bottom of the tube also became organge-red colored but viscous. Potatoes in their form were never destroyed into small pieces.

(vii) Litmus milk[5] : Although bubbles were not produced in the culture in litmas milk, on the third day casein was coagulated in the form of a clot, but not peptonized. Litmus underwent reduction and casein clots were colored yellowish orange. The culture in glucose-added milk medium exhibited active fermentation, but its external appearance after fermentation was the same as observed in the litmus milk medium.

(viii) Liquefaction of coagulated egg white : Active growth was seen by the inoculation with heat coagulated egg white pieces in the Starkey's medium. The cells were mostly grown on the egg white, and the sediment was scarecely produced. The egg white was intensively colored to orange-red and remarkably shrinked, but it was never liquefied.

(ix) Gelatin medium[5] : When a drop of the cell suspension of the organism was added to the surface of Starkey's medium containing 20 per cent gelatin and left at room temperature, gelatin was gradually liquefied from the upper part of the culture. Both the cultured and the remaining portions of the medium were not colored.

When the cells were cultured at 30°C in the gelatin medium converted into sols, a complete liqueaction of gelatin was well observable even after three days incubation.

4. Physiological characters :

(i) Utilization of carbohydrates : The media were prepared with the addition of 0.3 per cent peptone to the Starkey's medium, while glucose in the Starkey's medium was substituted with different carbon sources. In this case, organic acids, alcohol and acetone other than carbohydrates were also researched on their utility and each was added to the medium in 0.5 per cent. The observation was done during the five days and the fermented solution was analysed. The results then obtained were summerized in Table 2 together with those of the utilization experiment of nitrogen sources. As shown clearly in the table, neither fermentation or utilization was observed in the tests of pentoses, lactose, cellulose, alcohol and organic acids. After the completion of growth there produced the

orange-red colored pigment and viscous matters.

Table 2. Growth response using various carbon and nitrogen source.

Mono Saccharides	Growth*	Poly Saccharides	Growth*	Others	Growth*	Nitrogens Compounds	Growth**
xylose.	−	maltose	++	mannitol	++	ammonium sulfate	+
arabinose	−	lactose	−	glycerol	−	sodium nitrate	+
glucose	+++	sucrose	+++	ethanol	−	sodium nitrite	−
fructose	+++	raffinose	+	acetone	−	casamino acid	++
mannose	+++	starch	+++	acetic acid	−	casein	+++
galactose	+	glycogen	+++	lactic acid	−	peptone	+++
rhamnose	−	inulin	+++	succinic acid	−	urea	+++
		pectin	±			molecular nitrogen	+
		cellulose	−				

* Peptone was used as a nitorgen source.

** Glucose was used as a carbon source.

Productions of butanol and ethanol from available carbon sources observed when the fermented liquors were analysed, and a report of the analytical results will be described in detail in the forthcoming paper.

(ii) Utilization of nitrogen sources: The media were prepared from the Starkey's medium to which respective nitrogen sources were added in 30 mg per cent nitrogen. As shown in Table 2, utilization of nitrogen compounds was observed in all the cases except nitrite. An aerobic nitrogen fixer azotobacter species is occassionally known to utilize a certain organic nitrogen compounds, but general utilization of organic nitrogen source by this species is still questionable. The present result which showed a more utilizable character of organic nitrogen compounds for this anaerobic nitrogen fixer than inorganic ones was of great interest.

(iii) Indol formation: Plane peptone broth to which 7 per cent of the pre-incubated culture solution had been inoculated was incubated at 28°C for 48 hours, where the development of the bacterial growth though a large inoculum size employed was hardly seen. The experiment by the Ehlrich-Böme method was also negative.

(iv) Nitrate reduction: 0.1 per cent potassium nitrate-added peptone broth was inoculated by the same treatment as in c), and incubated at 37°C for 5 days, where no development of the bacterial growth took place likewise. However, the detection of nitrite by the use of the Griwss reagent showed clear coloration.

(v) Experiments on anaerobisity: Employing the *Cl. pasteurianum* medium containing 0.3 per cent peptone, the experiment was made by substitution of the gas mixture composed of carbon-dioxide and oxygen at different ratios. The test tube was held aslant to enforce the effect of the gas, and the volume of the solution was fixed at five ml.

As shown in Table 3, the growth was initiated even under the existence of 10 per cent oxygen and was also seen at the bottom of the tube even when substituted with 20 per cent oxygen and 80 per cent carbone dioxide. However, as the initiation of growth was not shown in case of the tight enclosure without the gas substitution, this strain was determined as anaerobic bacterium. Although carbon dioxide is considered to restore the impediment of oxygen to a certain extent, this problem will be investigated later.

Table 3. Growth response under various oxygen pressures.

CO_2 : O_2	Growth
100 : 0	±
	+
	+
95 : 5	+
	+
	+
90 : 10	+
	+
	+
80 : 20	±
	−
	−
70 : 30	−
	−
	−
50 : 50	−
	−
	−
0 : 100	−
	−
	−
vaccum	+
	+
	+

(vi) Fermentation products : As listed in Table 1, the products consisted of butanol as a main product and ethanol as a substantial product, while acetone and volatile fatty acids in negligible amount. Besides, iso-propanol was also formed in considerable amounts. It was almost characteristically observed so far tested that there occurred only a faint transformation into acids production during the fermentation process although it is generally recognized in acetone-butanol producing bacteria, and thus the acids forming period was quite indistinctive.

IV. Discussion

The anaerobic nitrogen fixing bacterial strain isolated from the paddy soil in the rhizosphere of the rice is characterized especially by its formation of orange-red pigment and clearly belongs to the genus Clostridium as based on its morphological and physiological characters. The characters of the strain are summarized in Table 4. In reference to Bergey's Mannual[6] and Prévot's Mannual[7], its analogous species can be cited as follows : *Cl. chromogenes* Prévot, *Cl. roseum*, McCoy and McClung, *Cl. felsineum* (Carbon and Tombolato), Bergey *et al.*, *Cl. flavum* McClung and McCoy, *Cl. aurantibutyricum*, Hellinger.

Cl. chromogenes diffuses red-colored pigments into the upper layer of the medium after the liquefaction of gelatin where whitish gray color of sediment then formed becomes red to violet-red. It produces grayish yellow-colored pigments on the potato slant, and also produces acids and gas from fructose, galactose, sucrose, lactose, maltose and mannitol. However, the tested isolate can not produce any pigments in the gelatin medium and becomes distinctly orange-red colored on potato slant and the orange-red pigmentation is not

Table 4. Physiological and morphological characteristics of the tested organism.

1. Litmus milk : Spongy clot was formed slowly without fermentation. When added with glucose, strong coagulation and fermentation occurred. Spongy clot was not digested.
2. Potato slant : Luxuriant growth. Potato was colored clear or range. turned brown when aged. Slime was not produced. Digestion did not take place.
3. Coagulated egg-white : Digestion did not take place but shrinking was observed.
4. Gelatin : Complete liquefaction was observed in 3 days. Sediment and supernatant were colorless.
5. Indol : Not produced.
6. Nitrate : Reduced to nitrite.
7. Vegetative cell : Rod, $1\sim2 \times 5\sim6\,\mu$. Usually single sometimes in pair, actively motile with peritrichous flagella, when old, became spindle formed colstridium.
8. Spore : $1.5 \times 2\,\mu$. Ellipsoidal, terminal or subterminal. Resistant 100°C 1 minute's heating.
9. Staining : Gram positive. Granulose negative.
10. Acid and Gas from glucose, fructose, mannose, maltose, sucrose, starch, glycogen, inulin, mannitol. Xylose, arabinose, rhamnose, lactose, cellulose, glycerol, ethanol, acetone, acetate, lactate, succinate are not fermented.
11. Products of Glucose Fermentation : Main product is butanol. Ethanol and iso-propanol are considerable amount. Acetone, atcetic and butyric acids are negligible.

increased on addition of chlorine or bromine water. Further, the strain is enable to utilize lactose. In these respects, the strain is quite different from *Cl. chromogenes*.

Cl. roseum produces orange-red pigments which became purplish black on aeration, coagulates litmus milk stormy, and rapidly digests potato to clear the yellow-colored fluid and where bluish sediments are formed. It utilizes xylose, arabinose, glucose, mannose, fructose, galactose, lactose, maltose, sucrose, raffinose, starch, dextrin, glycogen, inulin, pectin and salicin, but can not utilize mannitol. Whereas, the tested strain coagulates the litmus milk very slowly and does not carry out the digestion of potato. It can not utilize pentose and lactose, but can utilize mannitol. Then, our strain is also different from *Cl. roseum*.

Cl. felsineum produces orange-colored pigments which is not changed by aeration but changed to brownish by aging. In a resemblance to the isolated strain, it can utilize glucose, mannose, galactose, lactose, maltose, sucrose, dextrin, glycogen, inulin, pectin, and starch, being capable of producing solvents from those carbohydrates. However, *Cl. felsineum* can digest potato, forming a yellow slime and utilize lactos and pentoses, but it can not ferment mannitol. Besides, the formed solvents contain a considerable amount of acetone. These respects are reasonably mentioned as distinctive from the tested strain.

Cl. flavum coagulates litmas milk, and utilizes glucose, galactose, maltose,

sucrose lactose, pectin and starch, but can not ferment inulin and mannitol. Whereas, the tested strain shows a scanty coagulation of milk and also can not utilize lactose, but can ferment inulin and mannitol. Then the tested strain does not agree with *Cl. flavum*.

Cl. aurantibutyricum is also different from the isolated strain in the kinds of utilizable sugars and the fermentation products, though it considerably resembles to the latter in several respects. It utilizes xylose, galactose, glucose, sucrose, lactose, maltose, pectin and starch, but does not ferment inulin and mannitol. Whereas, the tested strain functions a notable utilization of inulin and mannitol as above mentioned but can not utilize xylose and lactose. In addition, the products of *Cl. aurantibutyricum* are comprised chiefly of acetic acid and butyric acids, while only a small amount of the solvents are produced as accessory products. On the contrary, the isolated strain are comprised chiefly of solvents such as butanol and ethanol, and volatile fatty acids are produced in a very small amount as accessory products.

In comparison with characteristic properties of the known species of Clostridium capable of producing any pigment, the present strain isolated by the authors is quite distinctive in its nature, particularly in its utilization and fermentation capacities of sugars or its fermentation products. Then the present strain is well recognized as a representative of a new bacterial species which can distinctly produce orange-red pigments and also butanol from available sugars as a main fermentation product. It is quite reasonable to term this newly isolated strain *Clostridium aurantiacum* nov. spc.

Diagnosis of *Cl. aurantiacum* Suto, Namba et Furusaka nov. spc. are as follows :

Straight rods, with rounded ends, occurring singly and sometimes in pairs. The vegetative cells measure 1~2×5~6 microns. Spore 1.5×2 microns, ovoid, terminal or subterminal, swelling the cells to clostridia. Motile by means of peritrichous flagella. Granulose-negative with iodine solution. Gram-positive.

Gelatine : Gradual liquefaction. Whitish gray sediment, no pigmentation.

Glucose-gelatine : Complete liquefaction in 78 hours at 30°C. Whitishgray sediment, no pigmentation.

Glucose agar surface colonies (anaerobic) : Compact, tabular form, surface and periphery smooth.

Glucose agar deep colonies : Compact, lenticular and smooth. Agar fragmented by abundant gas.

Pigmentation (anaerobic) : Fermentation liquors orange-red, becoming brown when aged.

Litmus milk : Gradual coagulation, litmus reduced. Casein clot colored orange-red, and no visible digestion.

Potato (anaerobic) : Rapid growth and colored clear orange-red, turned to brown

when aged. Slime was not produced. Digestion not occurred. Much gas with butanol odor.

Peptone water : No growth.

Meize mash : Rapid growth and colored orange-red, but decrease the growth during transferring.

Coagulated egg-white (anaerobic) : Digestion not occurred but shrinked, and colored orange-red.

Indol not produced.

Nitrate reduced to nitrite.

Acid and gas from glucose, fructose, mannose, maltose, sucrose, starch, glycogen, inulin and mannitol. Xylose, arabinose, rhamnose, lactose, cellulose, glycerol, ethanol, acetone, acetate, lactate and succinate were not fermented.

Anaerobic.

Optimum temperature : About 30°C but grows between 20–37°C.

Fermentation products are butyl, ethyl, iso-propyl alcohol, acetone, acetic, butyric acid and gas.

Atmospheric nitrogen fixed.

Distinctive characters (differentiation from Clostridium chromogeneses, Prévot, Clostridium roseum McCoy and McClung, Clostridium felsineum, (Carbone and Tombolato) Bergey et al., Clostridium flavum, McClung and McCoy, and Clostridium aurantibutyricum, Hellinger). Inability to ferment lactose, mannitol, xylose and arabinose ; failure to digest potato tissue. Production of orenge-red pigment and colored to brown when aged. Nitrogen fixation.

Source : Isolated from paddy soil.

Habitat : Soil.

Provided that the white-colored strain analogous to *Cl. aurantiacum* in its properties except pigmentation is a variety of this new species, Bergey's classification of the genus Clostridum dividing it into two major groups by the pigment productivity should be argued. However, the authors wish to their further investigations as to whether an interconversion between the colored and uncolored strains.

The existence of a considerable quantity of anaerobic nitrogen fixing bacteria in paddy soils throughout a year was evidenced by the results of the pot experiment during two years. Further, it is also noteworthy that there occurred a periodic change in those bacterial populations scuh as a periodic occurrence of the present solvent producing bacteria, as already mentioned. The comprise of nitrogen fixers in a number of the solvents producing *Clostridium* has been pointed out by Rosenblum.[8] Then, the bacteria of such type may possibly act a certain role in the nitrogen circulation in paddy soils. Further precise researches as to this concept may naturally be required.

V. Summary

1. When the transition of anaerobic nitrogen fixing bacteria of *Cl. pasteurianum* type was investigated in pot soils with rice plants in the fixed temperature glass room, anaerobic nitrogen fixing bacteria which produced orange-red pigments was found in about the same season throughout two consecutive years.

2. Those bacteria were capable of forming the solvents which comprised of butanol mainly, ethanol substantially, and a small amount of acetone, iso-propanol, and volatile fatty acids. Several diagnostic characters of those strains were investigated.

3. The bacteria produce orange-red colored pigment as well as viscous matters in a range of liquid media. They are well grown in the media containing nitrogen compounds rather than nitrogen gas, and utilized the organic nitrogen compounds to higher extent than the inorganic one. The bacteria are rod of $1\sim2\times5\sim6\,\mu$ in size, actively motile, possessed flagella and Gram positive. With the lapse of time, the bacteria became spindle shaped and belongs to *Clostridium* which produce oval spores $1.5\times2\,\mu$ at the terminal or subterminal.

4. The bacteria utilize glucose, fructose, mannose, maltose, saccharose, pectin, glycogen, inulin, starch and mannitol whereby they produce solvents, acids and gas. However, the bacteria do not utilize xylose, arabinose, rhamnose, lactose, cellulose, glycerol, ethanol, acetone, lactate and succinate. The coagulation of litmus milk is extremely slow, where the fermentation is hardly seen. Gelatin is liquefied, but the coagulated egg white is not digested. The bacteria is well grown on the potato slant, neither produce viscous matters nor liquefied potatoes.

5. In comparison with diagnostic properties of the known analogous bacteria, the authors named *Clostridium aurantiacum* as a new species.

We are especially grateful to Prof. Tenjiro Uemura for his interesting advise, and indebted to Dr. M. Fukushi for electron microscopic photography and to Dr. M. Suzuki for soil sampling. Th s experiment has been supported in part by grant from the Ministry of Education.

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Photo 1. Starkey medium containing peptone.
24 hrs. culture. Cr-shadowed ×10.000

Photo 2. Same medium.
48 hrs. culture. Cr-shadowed ×10.000

Studies on the Fermentation Products of *Clostridium aurantiacum*

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and Choseki Furusaka

(Received on March 1, 1960)

I. Introduction

In the course of the research of nitrogen fixing anaerobes in paddy soils, the authors could isolate a strain of Clostridium which formed orange-red colored aggreates and produced a considerable amount of viscous matters. As reported in the previous paper[1], this strain was named *Clostridium aurantiacum* based on its characteristic properties distinctive from other *Clostridium* species.

This strain is negligible in the accumulation of acetone, while produces butanol at a considerably high concentrations. Thus, the strain is of great interest from the industrial point of view. This paper deals with more detailed investigations on the fermentation products by this organism and periodic changes of its fermentation process when supplied with various kinds of carbon and nitrogen sources.

II. Experiment

Bacterial strains: As in the previous paper[1], *Clostridum aurantiacum* strains 0 (wild strain) and 0–1 (its naturally occurring, white colored mutant) were employed.

Culture: The media previously described were also used in this work composed chiefly of *C. pasteurianum* salts and Starkey's salts to which 0.3 per cent peptone was added in case of the carbon utilizing experiment while 2 per cent glucose in case of the nitrogen utilizing experiment. Except a large scale cultivation test, the media were usually poured into the test tube at an amount of 5 to 10 ml, and sterilized for 10 minutes under the pressure of 15 lbs. Before experimentation, those were boiled to expel oxygen, inoculated with one drop of the cultured solution and incubated at 30°C under reduced pressures (10 mmHg).

For the carbon source test, glucose, fructose, mannose, maltose, sucrose, raffinose, inulin, and mannitol each was added to the basal medium in 2 per cent whereas starch and glycogen in 1 per cent and pectin in 0.5 per cent, respectively. For the nitrogen source test, ammonium sulfate, sodium nitrate, peptone, casamino acid and urea, respectively, were added to the basal medium to an extent of 30 mg per cent nitrogen.

The 226th report of the Institute for Agricultural Research, Tohoku University.

Methods for analysis: The added and consumed glucose were analyzed by Somogyi's method.[2] Volatile fatty acids[3] (V.F.A.'s) were diffused into N/50 sodium-citrate in Conway's microdiffusion dish, and titrated with N/50 sodium hydroxide using cresol-red as indicator. Acetone[4] was diffused into the salicylaldehyde in Conway's microdiffusion dish and determined colorimetrically with the Hitachi photoelectric colorimeter at 470 mμ. The solvents produced were oxidized and then distilled according to the Johnson's method[5] and titrated with N/50 barium hydroxide using phenol red as indicator. For the quantitative analysis of isopropanol produced[6], the fermented liquors were subjected to the oxidation with bicromate-sulfuric acid mixture after neutralization and distillation, and acetone then formed was determined by the method described earlier.

Detailed analysis of the solvents produced was attempted by employing the apparatus of gas liquid chromatography technique with KGL-1 type of Hitachi Seisakusho, where the filler prepared from the mixture of glycerol with brick in a ratio of 10 : 4.5 (w/w) was plugged up in a column (4 mm in dia.) with the length of 3 meters. As the carrier gas, hydrogen was flowed at a rate of 30 ml/min., the filament was charged with electricity of 140 mA and the thermostat was adjusted at 78°C. For the purpose of dehydration, in case of a small quantity of the tested sample, 5 gr. of anhydrous potassium-carbonate and 0.5 to 0.3 ml of carbon tetrachloride or chloroform were used where the solvents tested were previously transfered into carbon tetrachloride or chloroform, and after the separation of it from the fermented liquor a small quantity of anhydrous potassium carbonate was further used for the completion of dehydration. Chromatography was carried out with the use of 20–30 μl of the above solution. Sometimes in this case, each peak of the solvens separated was too low due to its small amount to recognize. In the case of the use of 100–500 ml of the fermented liquor, it was neutralized and subjected to the repeated distillation up to the concentrations of 10–25 ml. The concentrated distillate underwent dehydration and the tested solvents was then transfered to chloroform or carbon tetrachloride according to the above mentioned method. The solvent-rich liquor thus obtained still contained water in relatively higher concentrations enough to cause an obstraction in the quantitative analysis by the gas liquid chromatography. Then an application of this technique for the quantitative analysis of solvents was given up and especially the treatment of dehydration for this purpose may require fruther investigations. In addition, as the peak of acetone partially doubled over the peak of chloroform, the area ratio accounted for acetone was deprived the portion caused by the extension of chloroform according to Keuleman's method.[7]

The platinum-saturated Calomel electrode was employed at 30°C for measurement of the oxidation-reduction potential, the value of which was reduced into pH 7 expressed with Eh_7[8].

The acetoacetate decarboxylation activity was measured by the Warburg

manometer (30°C) and the release of carbon dioxide from 10 μM acetoacetate was investigated in M/10 sodium hydroxide-citric acid buffer of pH 4.5.

III. Results

1. Fermentation products from various carbon sources

The tested carbon source and 0.3 per cent peptone were added to the salts medium of *Cl. pasteurianium*, to which 0 or 0–1 strains were inoculated, then cultured at 30°C for 5 days, and the fermentation products were analysed.

As shown in Table 1, those strains utilized each of glucose, mannose, maltose, saccharose, raffinose, starch and glycogen. Whenever fermentation took place, there was always observed the production of orange-red colored pigment, viscous matters and foams in the *Cl. aurantiacum* 0 strain. The final pH of every culture was within the range of 5.4–5.6 and the highest amount of the total volatile fatty acids produced attained only to the titration value of 0.3 ml/ml, not exceeding 0.3 mg where calculated as acetic acid, whereas a large amount of butanol (ca. 5 mg/ml). was produced from the well utilized carbon sources. The small amount of butanol production from starch and glycogen was accounted for their amount supplied. The amount of ethanol formed did not exceed 3 mg/ml, and that of acetone was also negligible, only being to the highest extent of 0.3 mg/ml. The ratio of the total solvents formed against each added carbon source was ranged in 35–40 per cent. In the case of strain 0–1 lacking the pigment productivity, its fermentation products were consisted chiefly of butanol showing no noteworthy difference in this respect.

Table 1. Fermentation products by *Clostridium aurantiacum* stranin 0 or strain 0–1 adding when various carbon sources added.

Strain 0	Carbon source.	Amt. supplied mg/ml.	pH	V. F. A. (ml. of N/50 $Ba(OH)_2$)	Bu-OH mg/ml.	Et-OH mg/ml.	Acetone mg/ml.	Total solvents. mg/ml.
〃	Glucose	20	5.4	0.14	4.73	2.73	0.2	7.66
〃	Fructose	20	5.4	—	2.60	0.30	—	—
〃	Mannose	20	5.4	0.32	5.29	2.15	—	7.44
〃	Maltose	20	5.4	0.22	4.79	1.54	0.3	6.61
〃	Sucrose	20	5.4	0.17	4.61	2.89	—	7.50
〃	Raffinose	20	5.4	0.20	4.90	2.70	0.1	7.70
〃	Starch	10	5.4	0.21	2.29	0.88	0.2	3.27
〃	Glycogen	10	5.4	0.20	2.49	1.25	0.1	3.80
〃	Inulin	20	5.4	—	2.10	0.20	—	—
〃	Pectin	5	5.4	0.20	0.59	0.92	—	1.51
〃	Mannitol	20	5.4	0.30	—	—	0.1	—
0–1	Glucose	20	5.4	0.22	4.52	2.64	—	7.16
〃	Starch	10	5.4	0.59	2.95	1.35	0.2	4.50

(Peptone was used as a nitrogen source)

2. Fermentation products from various nitrogen sources

The tested nitrogen sources were added to the *Cl. pasteurianum* medium so as to consist of 30 mg per cent nitrogen, which was, after inoculation, incubated

Table 2. Fermentation products by *Clostridium aurantiacum* strain 0.

Nitrogen source	Glucose Added. mg/ml.	Glucose Consumed. mg/ml.	Ratio of glucose. consumed %	pH	V. F. A. ml. of N/50 $Ba(OH)_2$	Bu-OH mg/ml.	Ef-OH mg/ml.
Ammonium sulfate	20	9.9	49.5	5.4	0.08	1.34	0.49
Sodium Nitrate	20	11.7	58.5	5.4	0.14	1.71	0.63
Casamino acid	20	16.8	84.0	5.4	0.20	3.45	1.94
Peptone	20	19.8	99.0	5.4	0.13	4.46	1.16
Urea	20	19.9	99.5	5.4	0.20	4.58	0.87

at 30°C for 5 days and the fermentation products were analysed.

As shown in Table 2, the inorganic nitrogen compounds tested did not facilitate the fermentation and caused a smaller production of butanol as compared with the organic ones. For example, ammonium sulfate or sodium nitrate, the amount of glucose consumed in each amounted to 50 per cent or so, where the formation of volatile fatty acids was at the titration value of 0.1 ml/ml, and the production of the solvents attained only to 1/2–1/3 of the quantity in the case of the use of organic nitrogen. On the contrary, the organic nitrogen could afford a nearly complete consumption of sugar and especially an increase of the butanol quantity (4–5 mg/ml) among the solvents produced. In this case the ratio of butanol to the total solvents was easily attainable to 28–30%, whereas it was 20% or so in case of the inorganic nitrogen.

3. Formentation course

Generally the fermentation process caused by solvents producing *Clostridium* is divided into three distinctive stages. It was then investigated whether this was also the case in the tested strains of new *Clostridium* species. The organisms

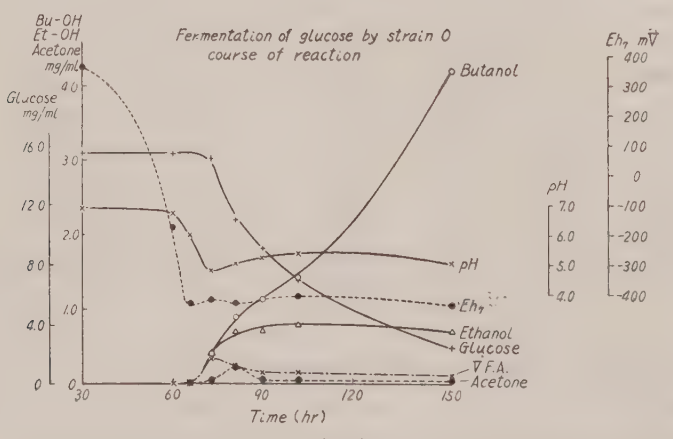

Fig. 1

When various nitrogen Sources added.

Acetone mg/ml.	Total Solvents mg/ml.	Total Solvents / Glucoe. consumed %
0.10	1.93	19.5
0.10	2.44	20.8
0.30	4.69	27.9
0.20	5.82	29.4
0.10	5.50	27.9

were inoculated into 3 l of *Cl. pasteurianum* medium containing peptone (0.3 per cent), and after culture at 30°C the sample taken periodically were analysed. As shown in Figure 1 and 2, either of the experimental strains showed initiation of the sugar consumption of only after a considerably long lag period raning 30–60 hours. Then pH of the media was depressed toward 4.8 along the sugar consumption, whereas againg ascended to 5.2–5.4 at the later growth stage. The production of volatile fatty acids was manifested with the fall of pH, but the amuont then produced was far smaller than by the strains of *Cl. acetobutylicum* type. In view of the fall of pH and the production of volatile fatty acids though both occurred a little, it became clear that either of the experimented strains was in possession of the acids producing period. On the other hand, the oxidation-reduction potential (Eh_7) decreased gradually at the beginning of the growing stage, and showed the lowest value (Eh_7 : about –400 mV) at the beginning of acids accumulation and was almost equilibrated even after the entry into the solvents stage. This potential is expressed 0.7 in rH value. The production of acetone attained to into maximum value (0.2 mg/ml) a little later than the period of the maximum production of volatile acids, and then was decreased with the lapse of time. This fact may suggest that either acetone thus formed would again enter the metabolic cycle of fatty acids with the re-fixation of carbon dioxide, or it may undergo its reduction into iso-propanol. From the results later described, it was presumable that the latter conversion of acteone into iso-propanol would be the case. In view of the increase of butanol and ethanol after the acids producing period, it

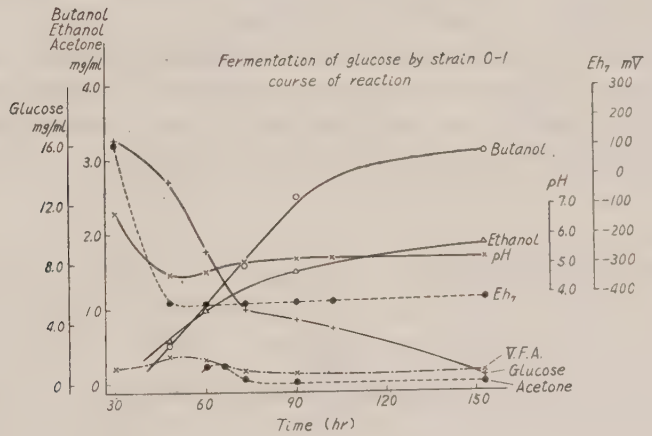

Fig. 2

was made clear well mentioned that there was though indistinctive the acids reducing period during the fermentation course by the experimented strains.

4. Quantitative analysis of iso-propanol

As mentioned above, the formation of iso-propanol was predicted by the changes of acetone during the fermentation process. Accordingly, the authors investigated firstly whether an increase of acetone could be caused by the oxidation with bicromate-sulfuric acid mixture of the solvents contained in the fermentated liquor. The O strain was inoculated into the *Cl. pasteurianum* medium containing 0.3 per cent petone, and after culture at 30°C for 153 hours, the fermented liqur was directly subjected to the quantitative analysis and the amount of acetone contained in the liquor was showed 0.08 mg/ml in Table 3. Further, after neutralization of fermented liquor, the quantity of acetone contained in the distillate showed 0.083 mg/ml, being approximately the same as the result obtained before distillation. When the distillate above obtained was oxidized with bicromate-sulfuric acid mixture and the resultant acetone was measured, its quantity amounted to 0.8 mg/ml.

Table 3. Analysis of iso-propanol contents in fermented liquor.

	mg/ml.
In peptone medium	0.080
In distilate after neutralikation	0.083
" after acid-chromate oxidation	0.80

This increment of acetone may well be accounted for the presence of iso-propanol in the fermented liquor, and thus a consibreable amount of isopropanol may be formed during the fermentation by this organism.

5. Analysis of the solvents by gas liquid chromatograpny

As the presence of iso-propanol was not confirmed by the paper chromatogarphy based on the oxidation with bicromate-sulfuric acid mixture and conversion of the resultant fatty acids to hydroxamic acid, the fermentation solution was further analysed by the gas liquid chromatography.

As shown in Figure 3, the peaks were well recognized in acetone, iso-propanol, ethanol and butanol other than chloroform. As regards the ratio of each component calculated from its occupied peak area was as follows ; Relative acetone 0.5%, iso-porpnaol 25.6%, ethanol 7.4 and butanol 66.5%.

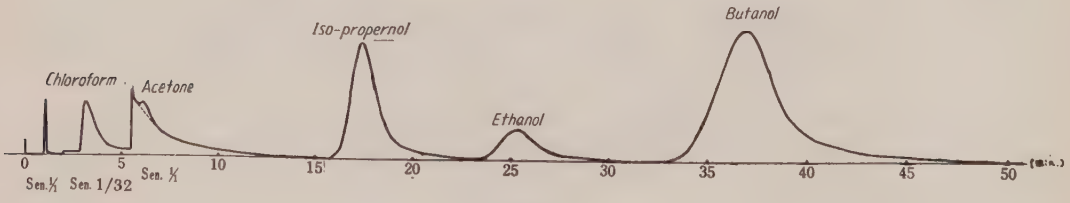

Fig. 3

6. Measurement of acetoacetatedecarboxylation activity

As mentioned above, a small quantity of acetone was always existent in the fermented liquor, and iso-propanol seemingly derived from acetone was comparatively abundant, and also the formation of acetone was usually observed at a later period than the acids producing stage as in the case of acetone-butanol bacteria. Those facts suggested an existence of acetoacetate decarboxylase in cells of the tested organism the irrespective of a scanty acetone production observed. Cells of strain O and strain O–1 of *Cl. aurantiacum* were collected from their cultures at for media containing peptone washed two times and then the release of CO_2 from the added substrate was manometrically estimated with those washed cell supsensions. Both strains showed a notable activity as being tabulated, where the values obtained approximated almost to the activity of the ripe cells of *Cl. acetobutylicum* No. 314. This may lead to an assumption that those tested strains may form acetone rather actively and most of which, however, may be reduced into iso-propanol, and thus a scanty acetone production, may be effected.

Table 4. Acetoacetate decarboxylase activity of Cl. aurantiacum compared with Cl. aceto butylicum No. 314.

		Culture	$Q^N_{CO_2}$
		hr.	
Cl. aurantiacum	Strain O	20	1950
〃	〃 O–1	48	835
Cl. acetobutylicum	No. 314	9	435
	〃	21	796
	〃	32	2100
	〃	51	646

IV. Discussion

In the results of the analysis of the fermented liquor according to Johnson's method, the solvent obtained was composed of 5 mg of butanol, 2–3 mg of ethanol, 0.7 mg of iso-propanol (later growth stage) and 0.2 mg of acetone, per ml, the relative molar ratio of the solvent formed against the consumption of glucose being 38–45%. The analytical result obtained by the gas liquid chromatography showed acetone ; 0.5%, ethanol ; 7.4%, iso-propanol; 25.6%, and butanol ; 66.5%, and this result indicates a considerable difference from the analytical values above cited. This difference may be caused by more inadequate treatment such so oxidation procedure in Johnson's method, and will be studied in future. The ratio of the solvent formed against glucose consumed is 30–35% in the case of *Cl. acetobutylcum*, No. 314 while the authors' strains produced such a higher yield of the solvent as 30–40%. In the case of *Cl. acetobtylicum* known to be one of the typical organisms as the acetone-butanol forming bacteria, the ratio of acetone : butanol : ethanol is usally 10 : 20 : 3, and other analgous bacteria for has been shown nearly the same ratio as above mentioned. On the other hand, several kinds of sugar fermenting *Clostridium* species are well known to produce iso-propanol. For example, in *Cl. saccharo-isopropyl acetonicum-beta* the ratio of acetone : butanol : ethanol : isopropanol was 15–40 : 60–85 : – : 0.1–4.0, in *Cl. toaum* 4–11 : 53–65 : 0.4–5.6 : 29–39, and in *Cl.*

butanologenum 4.9 : 6.3 : 7.2 : 24.4, respectively. In the author's strains *Cl. aurantiacum*, the ratio was 0.5 : 66.5 : 7.4 : 25.6, and thus the strain may be regarded as a butanol-iso-propanol producer.

Cl. aurantiacum also showed the same fermentation process as the solvent producing species such as *Cl. acetobutylicum* and had both the acids producing and the acids reducing preiods. However, both the periods were not so distinct in the former organism as in the latter one, although a successible change between both periods, was all detectable in the former organism, the oxidation-reduction potential showed the lowest value at the acid producing periods, and underwent the solvent producing period. Periodic changes of the redox potentials during the fermentation process also has been often investigated by several workers with the use of acetone-butanol bacteria, it was usually mentioned that the first drop of the potential occured at the acid producing period is soon followed by its gradual accordance up to a little peak at the acid reducing period at the fermentation process by strain of *Cl. aurantiacum* such a little peak of potential variations was decerned, and this may be explained by the fact of scanty production by the organism which indicates sooner conversion of the acid form to the solvents.

In view of the decrease of acetone once formed with the lapse of time and the accumulation of iso-propanol in the media at the later stage, it is conceivable that the formed acetone is reduced successively to iso-propanol. As already mentioned, the fact that the organism showed a considerable activity of acetacetate decarboxylation as may be well assessed in this respect.

Dr. Bunzo Rokusho and his collaborators have recently investigated the practical use of this new species for an industrial butanol production, and formed, though an available butanol producer, a range of shortages in this organism such as its long lag period or its weak sugar-tolerance. However, devising fermentation conditions in several respects, they have succeeded in employing strain for the butanol production in such a large scale as of 200 tones of fermentation mash, where a considerable amount of iso-propanol was actually formed during the distillation process.

V. Summary

1. *Cl. aurantiacum* strains 0 and 0–1 produce a large amount of butanol as the main fermentation product when various carbon and nitrogen sources were used.

2. The analysis of the solvents produced showed a comprizal of iso-propanol in addition to butanol, ethanol and acetone.

3. Those bacteria have both the acids forming period and the acids reducing period along the development of their growth as in the case of the solvent producing *Clostridium*, but the acids forming period is not explicit.

4. Those bacteria renoted with active acetoacetate decarboxylase, despite

of the fact that the accumulation of acetone is not remarkable in their fermentation.

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